tively) are composed of a mixture of DNA and polyadenylic acid. This result would be anticipated from the data shown in Fig. 1 (DNA alone) and in Fig. 3A (polyadenylic acid alone). The values A_{280}/A_{260} of fractions included in peaks 4, 5 and 6 are scattered within the limits of 0.48 ± 0.06. The A_{280}/A_{260} value for a solution of this polyadenylic acid of like concentration is 0.32 and for this sample of DNA, 0.54. No DNA was found in fractions of peaks 1, 2 and 3. In fractions of peaks 4, 5 and 6 72% of the DNA originally in the mixture placed on the column was recovered. This was distributed as follows: 25.8% in peak 4, 36.7% in peak 5, and 9.4% in peak 6.

Discussion

To be of use for the separation and purification of macromolecules, a calcium phosphate adsorbent must be able to adsorb and to desorb such molecules under the influence of appropriate eluents. For this adsorbent to be useful for purposes of *column* chromatography, the individual particles must be of sufficient size and/or favorable shape as to allow adequate eluent flow rates through columns of the same. In contrast to the coarser crystal size of acid and neutral calcium phosphates, small crystal size is a striking feature of the chemically precipitated basic calcium phosphates and apatites.[35] Both the average crystal diameter (range 150–300 Å.)[36] and the fine flat needle-like external structure[37] of precipitated calcium apatites favor such dense packing as to prevent adequate flow rates through columns of these crystals. The electron microphotographs of Hayek, *et al.*,[37] indicate that acid precipitated ($CaHPO_4$) calcium phosphate crystals[38] are much coarser grained by comparison with the above and in external form are triclinic approaching a cuboidal form. Tiselius and coworkers,[3] circumvented these unfavorable conditions by converting preformed coarser-grained brushite ($CaHPO_4 \cdot 2H_2O$) crystals into a semi-hydroxylapatite (approaching $Ca_{10}(PO_4)_6(OH)_2$) of particle size suitable for column chromatography. This was accomplished by boiling the former in NaOH solution, followed by washing and heating the crystals for various lengths of time in a series of phosphate buffers. This preparation of calcium phosphate has found application in the column chromatography of proteins by Tiselius[3] and by Li[39] and of DNA by Semenza.[11] The latter author was able to separate DNA (previously adsorbed in 0.05 *M* phosphate buffer +1.0 *M* NaCl) into two chromatographic peaks, desorbed, respectively, by 0.30 and by 0.35 *M* phosphate buffer, *p*H 6.3, each in 1.0 *M* NaCl solution. Maximum resolution was obtained with a load of 0.25 mg. of DNA per column (16 × 50 mm.), which is 1/8 of the load resolved by the present authors' method.

Characteristics of this calcium phosphate preparation which recommend it for column chromatography are: ease and reproducibility of preparation; high eluent flow rates through packed columns; stability; and the degree of chromatographic resolution obtained, (a) at or near physiological concentrations (ionic strength and *p*H) of the eluents and (b) for the size of load placed on the column.

(35) J. J. Weikel, W. F. Neumann and I. Feldman, THIS JOURNAL, **76**, 5202 (1954).

(36) A. S. Posner, A. F. Doris and A. Perloff, *Natl. Bur. Standard Tech. Bull.*, **41**, 88 (1947).

(37) E. Hayek, F. Mullner and K. Noller, *Monat. Chem.*, **82**, 959 (1951).

(38) The present authors have refrained from applying the term "gel" ("(a) a jelly or (b) a solid gelatinous form in which a colloidal system is sometimes obtained as distinguished from the liquid form of sol"; ref., "Chambers Technical Dictionary," The Macmillan Co., New York, N. Y., 1956, p. 371; Van Nostrand Chemists Dictionary, D. Van Nostrand Co., Inc., New York, Toronto, London, 1953, p. 321, etc.), to any of these aqueous suspensions or packed preparations of calcium phosphate crystals.

(39) C. H. Li, *J. Biol. Chem.*, **229**, 157 (1957).

SAN FRANCISCO 24, CALIF.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGICAL CHEMISTRY, THE UNIVERSITY OF MICHIGAN, ANN ARBOR MICH.]

Cupric Chelates of Pyridoxylvaline and Pyridoxylidenevaline[1]

BY HALVOR N. CHRISTENSEN

RECEIVED JULY 6, 1959

These two chelates have been compared to clarify a previously observed association in the 3-hydroxypyridine system between (a) the formation of stable linkage to the phenolic oxygen and (b) a lowering of the pK_a of the pyridine N. The results show that the pK lowering depends upon the extent of electron displacement occasioned by the linkage and not upon its presence or absence. In Cu pyridoxylvaline, Cu is strongly linked to the phenolic O with only a small displacement of the pK; only upon deprotonation does the spectrum show a strong new deformation of the electron distribution.

Tentative structures attributed to metal chelates of pyridoxylidene amino acids showed the metal bonded to the Schiff base at 3 points (like Fig. 1, structure II), although alternative formulations left the carboxylate group free, probably to assist in explaining how *α-decarboxylation* can be catalyzed.[2] A study of a group of chelates of pyridoxylidenevaline led, however, to the conclusion that if any one of the 3 groups remains free, it will tend to be the phenolic group.[3] A downward shift of the pK_a of the pyridinium N by 3 or more *p*H units is believed to signal covalent linkage of the phenoxide ion to the metal (Cu(II), Fe(III)), eliminating the usual powerful inductive effect of the phenolic group of the 3-hydroxypyridines on the pyridinium dissociation. A small shift ((Ni-(II), Mn(II), Fe(II), Zn) was interpreted to repre-

(1) Supported in part by a grant (C4268-C3) from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service.

(2) D. E. Metzler, M. Ikawa and E. E. Snell, THIS JOURNAL, **76**, 648 (1954).

(3) H. N. Christensen, *ibid.*, **79**, 4073 (1957)

sent much weaker or absent bonding at this position.

For the zinc chelate this conclusion appeared to be particularly unequivocal, since an atom of the tetracovalent metal bound two pyridoxylidenevaline molecules, which restricted it to an average of two bonds to each ligand molecule.

Recently the substituted amino acid, pyridoxylvaline, obtained by hydrogenating the corresponding Schiff base, has been observed to form a 1:1 Cu(II) chelate with a pK_a only 0.7 unit below that shown by the pyridinium N of the free ligand molecule. This observation called for a reconsideration of the nature of the chelates of hexacovalent divalent metal ions.

Experimental

Pyridoxyl-L-valine.—The general procedure of Heyl, *et al.*,[4] admittedly was not well adapted to pyridoxyl-L-valine and pyridoxyl-L-leucine because of their excessive water solubility. Instead the valine Schiff base had been reduced in water solution in this way: 5 mmoles each of L-valine and pyridoxal were dissolved in 10 ml. of 0.50 *N* $Ba(OH)_2$ and the Schiff base hydrogenated in the presence of 30 mg. of Adams catalyst for 20 min. at 3 atmospheres total pressure. Then 10 ml. of 0.50 *N* H_2SO_4 was added gradually, to give a *p*H of 6.0. After standing overnight the solution was centrifuged clear and taken to dryness in a desiccator over $CaCl_2$. The dry residue was taken up quickly and completely in 200 ml. of warm methanol, from which it soon began to crystallize. Before this occurred the solution was concentrated to about 25 ml. The crystals separating were again crystallized under the same conditions with a 70% over-all yield.

The titration behavior of the product confirmed that the reaction had taken its expected course. pK_2' was determined spectrally (by the rise of both the 325 and 253 mμ peaks) and acidimetrically to be 3.10; pK_3' was found spectrally (by fall of density both at 395 and 330 mμ) and alkalimetrically to be 7.90; pK_4' was determined by titration to be about 10.6. Comparison with the spectral behavior of other 3-hydroxypyridines permits pK_2' to be identified with the phenolic group, pK_3' with the pyridinium group, leaving pK_4' for the secondary amino group. No spectral charge was recognized to be associated with the carboxylic titration.

Cupric Pyridoxyl-L-valine.—One tenth millimole of pyridoxylvaline was dissolved in 0.33 ml. of 0.3 *M* cupric acetate. Addition of 0.33 ml. of 0.3 *M* NaOH led to no crystallization upon standing (in contrast to the behavior of the Schiff-base chelate) but adding another 0.1 ml. of the alkali started heavy crystallization (clear green rectangular tablets). Later cautious addition of 0.1 ml. more NaOH increased the yield. The degree of titration yielding the maximal amount of pure product was not determined. After drying at 56° over P_2O_5 one mmole of pyridoxylvaline (by the absorbancy in 0.1 *M* HCl) was found in 327 mg., and one mg.-atom of Cu[5] in 325 mg. The theoretical molecular weight is 330.

One can accelerate the separation of the Cu-chelated Schiff-bases of various amino acids also by adding part or all of the second equivalent of alkali theoretically required to form the neutral chelate but the products tend to be amorphous. Possibly hydrous cupric oxide precipitation interferes with crystallization. The standard procedure[3] permits the chelate to form and saturate the solution gradually in an acetate buffer, donating its H^+ to the acetate as it crystallizes.

Spectral and Titration Studies.—Cu pyridoxylidenevaline was used in crystalline form, whereas Cu pyridoxylvaline was generated in aqueous solution by combining equimolar quantities of the components. Visual spectra were studied at 0.001 to 0.02 *M*, ultraviolet spectra at 0.0002 *M*. The recorded spectral observations were made with a Zeiss UV spectrophotometer at 20°, although *p*H determinations were made at 25°. Acetate buffers were used from *p*H 4 to 6, HCO_3^-/H_2CO_3 or CO_2^{--}/HCO_3^- systems from *p*H 7 to 10, all at 0.05 *M*, using $KClO_4$ to bring the ionic strength to 0.10.

In the spectral determination of the hydrogen ion dissociation of Cu pyridoxylvaline, the solution was self-buffered at a 0.004 *M* level at $\Gamma/2 = 0.10$. For the time-studies of Cu pyridoxylidenevaline formation at 0.02 *M* concentration the acetate buffer was raised to a 0.20 *M* level.

(4) D. Heyl, S. A. Harris and K. Folkers, THIS JOURNAL, **70**, 3429 (1948).

(5) A. J. Woiwod, *Biochem. J.*, **45**, 412 (1949).

Results and Discussion

For discussion Cu pyridoxylidenevaline will be designated as the *Schiff-base chelate*, Cu pyridoxylvaline as the *amino acid chelate*. Two significant contrasts between these two are the following.

1. The titration of the amino acid chelate shows a pK_a' of 7.22 (25°, $\Gamma/_2 = 0.10$) compared with the corresponding $pK_3' = 7.90$ for the organic ligand. Hence the presence of the metallic ion lowers the pK' only by 0.68, indicating a minimal displacement of electrons around the pyridinium N by metal interaction with the ring. In contrast the pK' of the Schiff-base chelate is diminished by about 3 units from that applying to the pyridinium N in the absence of metal- or hydrogen-bonding to the phenolic group.[3]

2. Although deprotonation has similar effects on the peaks in the ultraviolet for the two chelates (Table I; *cf.* ref. 3), the spectrum of the amino acid chelate in the visual range is radically modified by deprotonation (Fig. 2) with an obvious color change from blue-green to yellow-green. The protonated form has a spectrum resembling the sum of the spectra for the organic ligand and Cu valine$^+$. Upon deprotonation a new peak appears at 395 mμ (A_m about 410) and the absorbancy is increased by 4 to 6 times between 400 and 500 mμ. Furthermore, the peak in the upper visual range shifts from 687 to 656 mμ and the absorbancy increases by $^1/_3$ (Fig. 2). Accordingly, only upon deprotonation does a radical electron redistribution appear to take place, of a character that appears already to have occurred when the chelate of the Schiff-base was first formed, whether in protonated form or not. The latter chelate shows only a small spectral change in the visual range on deprotonation and one cannot differentiate a solution at *p*H 5 from one at *p*H 8 by visual examination.

TABLE I

SPECTRAL BEHAVIOR OF PYRIDOXYLVALINE WITH *p*H[a]

In free state	λ_{max1}	A_m	λ_{max2}	A_m
*p*H 1.0	297	8700	..	..
*p*H 5.0	325	8400	251	4400
*p*H 9.5	308	6000	243	5700
*p*H 13	309	8100	244	7000
Cu pyridoxylvaline				
*p*H 5.0	315	6800	251	6800
*p*H 9.5	300	7750	242	7400

[a] Removal of H^+ from the phenolic group at $pK_2' = 3.10$ causes the peak of 297 to disappear and new ones at 325 and 251 to rise. Removal of H^+ from the pyridinium N, $pK_3' = 7.90$, cause both the latter peaks to shift to lower wave lengths. A further titratable group presumably represents H^+ removal from the secondary amino group and accentuates both of the existing peaks. Chelation with Cu(II) moves the peak above 300 mμ downward about 10 mμ, whether at *p*H 5.0 or 9.5. The other peak is intensified but scarcely shifted by chelation.

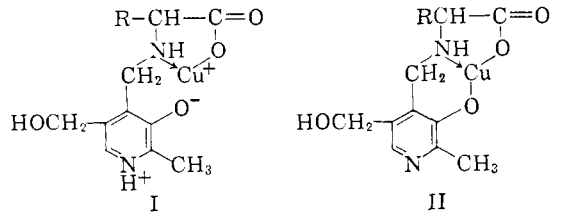


Fig. 1.—Possible stuctures of Cu pyridoxylvaline. As an extreme view the protonated form might be considered to have a completely ionic link (I) between Cu and the phenolic oxygen, since the electrons are displaced only slightly in the pyridine ring. The spectral change on deprotonation could then represent conversion of this link to covalent form (II). So categorical a view probably is not justified.

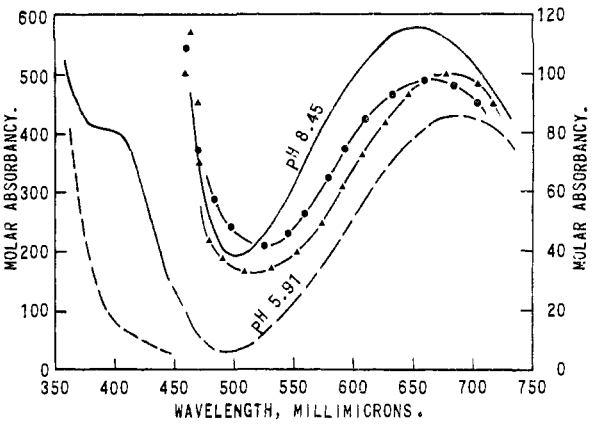


Fig. 2.—Effect of protonation on visual spectrum of Cu pyridoxylvaline and Cu pyridoxylidenevaline. The solid and dashed lines show the spectra of the amino acid chelate in its protonated and unprotonated forms, *p*H values as indicated. The two lines with inserted points show the spectra of the Schiff-base chelate at *p*H 5.1 (lower curve) and at *p*H 8.7. The absorbancy scale at the left applies below 450 mμicrons, the scale at the right above this wave length.

As a further contrast Cu pyridoxylvaline forms instantly, whereas the Schiff-base chelate requires over 24 hours at *p*H 5.6 at 0.02 *M* levels of the three components to reach a stable spectrum. This change does not represent transamination since pyridoxal is not lost, nor is it at pH 9.5 where the spectrum stabilizes in an hour. The binary products, Cu valine, Cu pyridoxal and pyridoxylidenevaline all are formed far more quickly. The possibility to be considered is that the Schiff-base chelate passes through an intermediate form resembling the protonated amino acid chelate, perhaps like structure I of Fig. 1, with minimal electron shifts in the pyridine ring, and that the rate-limiting reaction actually may be the subsequent formation of the covalent link to the phenolic oxygen, producing proton displacement. If one rapidly titrates an equimolar mixture of pyridoxal, $CuCl_2$ and valine with OH^-, the second equivalent shows an apparent titration mainly above *p*H 7.0, with the *p*H gradually falling to lower values. At 1.5 equivalents per mole added rapidly (2 are calculated to form the neutral chelate) a precipitate forms, but on immediate redissolving this product in a *p*H 5.6 acetate buffer, it shows the usual spectrum of the Schiff-base chelate. Hence, the presence of an intermediate chelate is not indicated. Small immediate spectral changes on adding pyridoxal to Cu valine$^+$ at *p*H 5.6 suggest some immediate interaction. Nevertheless, the slow reaction between the 3 components at 0.02 *M*, *p*H 5.6 and 20°, shows bimolecular kinetics, K = 0.19 l. mole^{-1} min.$^{-1}$, calculated from the rise in the 680 mμ absorbancy. Accordingly, if an intermediate 1:1:1 chelate exists its formation is small and rate-limiting.

As a possible interpretation of the shift of the spectrum of Cu pyridoxylvaline on deprotonation, the possibility was examined that Cu was largely unlinked to the phenolic O. If so, a second molecule of the amino acid ought to bind to the Cu, yielding Cu(pyridoxylvaline $H^+)_2$. Study by the Job method of continuous variation showed, however, maximal absorbancy at 680 mμ at the 1:1 composition at *p*H 5 as at *p*H 9.8. This was also the composition of the crystalline chelate. Even a 25:1 excess of the ligand did not modify significantly the molar absorbancy.

A similar consideration may be applied to Cu pyridoxamine, which shows a similarly high *pK'* and a large shift in the visual spectrum on deprotonation. Nevertheless, the protonated form has the composition Cu(pyridoxamine $H^+)_2$ as shown at *p*H 6.0 by Williams and Neiland[6] and also confirmed in this study for *p*H 5.0.

Accordingly, Cu must be linked to the phenolic oxygen in Cu pyridoxylvaline strongly enough to prevent its linkage to a second molecule of the amino acid. From the small effect on the *pK* this linkage must, however, have little effect on the electron distribution around the pyridine N. By older concepts one might describe the link as ionic, and the change upon deprotonation as a change to a covalent nature (Fig. 1). Without implying such a categorical change, one may say that upon deprotonation of the pyridinium N, Cu interacts with the phenolic oxygen to produce a new deformation of the electron distribution, and that a related degree of deformation appears to exist in Cu pyridoxylidenevaline even in the protonated form since a similar change in visual spectrum occurs mainly upon its formation in the protonated form, rather than upon its subsequent deprotonation.

This difference in protonated chelate structure does not arise from large differences in stability, a question raised earlier; in fact the amino acid chelate is almost as stable as the very stable Schiff-base chelate; when the latter is mixed with pyridoxylvaline, both at 3.3×10^{-4} *M* at *p*H 5.0, 40% of the copper is stolen by the latter, leading to an equivalent release of pyridoxal and presumably of valine. A similar destabilization of the Schiff-base chelate occurs at *p*H 9.15. No doubt the stabilizing influence of the double bond of the aldimine structure on the chelate ring is counterbalanced by the low stability of the aldimine link itself.

(6) V. R. Williams and J. B. Neiland, *Arch. Biochem. Biophys.*, **53**, 56 (1954).

These findings show that the absence of a depression of the pK' of the pyridine N does not establish the absence of bonding to the 3-phenolic group. The results do not mean, however, that the earlier conclusion as to the nature of M(pyridoxylidenevaline)$_2$, where M is Mn(II), Ni(II), Fe(II) or Zn(II), must be modified. The first 3, being hexacovalent, could form a link of predominantly ionic character to the phenolic oxygen without the presence of the link being betrayed by a low pK for the pyridine N. Such a linkage is unlikely, however, because these metals are already rendered neutral by the electrons received from the carboxylic oxygen. For zinc such bonding is particularly unlikely.

One of the strongest implications of a metal in a B_6-enzyme action has been found for a decarboxylation, namely of histidine.[7] If metals do participate and if we cannot anticipate that the phenolic oxygen will compete successfully with the α-carboxyl group for chelation to the metal, we must look to other groups to do so, since decarboxylation can hardly be expected for a carboxyl group involved in a 5-membered chelate ring. In this connection, apparently only amino acids having a third functional group are decarboxylated by B_6 enzymes. In histidine and aspartate, effective competition for chelation by the β-functional group with the α-carboxyl group may be anticipated. In the enzymatic reaction the hydrogen ion as a "chelator" might largely avoid this problem. Conceivably groups on the protein molecule could also help to release the α-carboxyl group from a chelate ring.

(7) B. M. Guirard and E. E. Snell, THIS JOURNAL, **76**, 4745 (1954).

The Cu chelate of the Schiff-base formed between pyridoxamine and pyruvate titrates with NaOH at about pH 6.3 in crude determinations eventually complicated by precipitation. Apparently the double bond does not need to lie inside the fused ring system to obtain a large downward shift of the pK (although understandably this chelate is much less stable, and also suffers spectral changes[8] that we find are due to extensive transamination, Cu-catalyzed autoöxidation of pyridoxamine to pyridoxal[9] and other reactions). Instead, the acidity of the imine N appears to determine how the Cu ion influences the acidity of the pyridine N. That is, an appropriate metal atom transfers the quality of acidity from the imine N to the pyridine N. Metals that show little of this effect are nevertheless catalytically quite effective in non-enzymatic transaminations.

Cell Permeability to Pyridoxylvaline, Pyridoxylglycine and 5-Phosphopyridoxylglycine.—These reduction products might be expected to have molecular shapes rather like those of the Schiff bases. Accordingly, Mr. Gene Sellers in this Laboratory, determined whether they enter Ehrlich ascites tumor cells readily, measuring their concentration in metaphosphoric acid extracts of cells by the absorbancy at 295 mμ. A positive finding might have important biological implications. As anticipated, however, these highly charged substances failed to enter the water of the cells to any significant extent during one hour at 37°.

(8) G. L. Eichhorn and J. W. Dawes, *ibid.*, **76**, 5663 (1954).
(9) D. E. Metzler and E. E. Snell, *ibid.*, **74**, 979 (1952).

[CONTRIBUTION FROM THE ORGANIC RESEARCH LABORATORIES, U. S. VITAMIN & PHARMACEUTICAL CORP.]

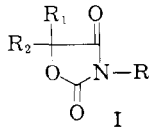
N-Substituted Oxazolidinediones

BY SEYMOUR L. SHAPIRO, IRA M. ROSE, FRANK C. TESTA, ERIC ROSKIN AND LOUIS FREEDMAN

RECEIVED MAY 21, 1959

A series of oxazolidine diones has been synthesized and examined for anticonvulsant activity, ultraviolet absorption characteristics and behavior upon alkaline hydrolysis.

Our investigations of oxazolidinediones with pharmacological activity[1] are herein extended to compounds of the type I. Such compounds and



R_1, R_2 = H, methyl, phenyl
R = alkyl, cycloalkyl, aralkyl, aryl

I

closely related systems have been inspected by others[2-7] and their synthesis has been reviewed.[8-10]

(1) (a) S. L. Shapiro, I. M. Rose, E. Roskin and L. Freedman, THIS JOURNAL, **80**, 1648 (1958); (b) **81**, 386 (1959); (c) S. L. Shapiro, I. M. Rose and L. Freedman, **81**, 3083 (1959); (d) S. L. Shapiro, I. M. Rose, F. C. Testa and L. Freedman, *ibid.*, **81**, 5646 (1959).
(2) M. A. Spielman, *ibid.*, **66**, 1244 (1944).
(3) M. A. Spielman and G. M. Everett, *ibid.*, **70**, 1021 (1948).
(4) E. Testa and R. Ettore, *Arch. Pharm.*, **290** [62], 532 (1957).
(5) C. A. Miller and L. M. Long, THIS JOURNAL, **73**, 4895 (1951), (succinimides).
(6) S. R. Safir and R. J. Lopresti, *ibid.*, **80**, 4921 (1958), (1,2,4-oxadiazolidinones).
(7) C. Chu and P. C. Teague, *J. Org. Chem.*, **23**, 1578 (1958), (hydantoins).

An extensive series of compounds of the type I was needed for consideration of the effect of structural variation on anticonvulsant activity, ultraviolet absorption spectra, and the pattern of alkaline hydrolysis of I to carbamoyloxyacids and α-hydroxyamides.

The compounds prepared have been described in Table I.

Synthesis.—To achieve the desired synthetic scope, the more familiar procedures involving reaction of I, R = H, with halides[9] or utilization of RNCO[8] as an initial reactant were not attractive, and a variety of other methods was evaluated.

The pyrolysis of carbonate esters of α-hydroxyamides[11] (method 1) proved to be a straightforward and effective procedure for obtaining I.

(8) R. F. Rekker, Thesis, University of Amsterdam, 1950.
(9) J. W. Clark-Lewis, *Chem. Revs.*, **58**, 63 (1958).
(10) R. C. Elderfield, "Heterocyclic Compounds," Vol. 5, John Wiley and Sons, Inc., New York, N. Y., 1957, p. 411.
(11) S. L. Shapiro, I. M. Rose and L. Freedman, THIS JOURNAL, **81**, 6322 (1959).