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The Preparation of Barium Monophosphotaurocyamine

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The synthesis and isolation of monophosphotaurocyamine (*N*-phosphoguanidoethanesulphonic acid) has not been described, although the compound is of interest as the naturally occurring phosphagen in certain annelids (van Thoai, Roche, Robin & van Thiem, 1953). These authors reported its isolation as a calcium salt after the phosphorylation of taurocyamine by phosphoryl chloride in an alkaline medium. A later communication (van Thoai & van Thiem, 1957*a*) reported the isolation of phosphotaurocyamine as a crystalline ammonium salt, but no details were given. In view of the difficulties which have been encountered by the present workers, because at least two products are formed in the phosphorylation reaction, it was considered worth while to report details of the synthesis and isolation.

This communication deals with the synthesis of taurocyamine, the products of the reaction between phosphoryl chloride and taurocyamine, the separation in pure form of barium monophosphotaurocyamine and some of its properties.

MATERIALS AND METHODS

Taurine (British Drug Houses Ltd.) was used without further purification.

Taurocyamine was estimated by the method described by Rosenberg, Ennor & Morrison (1956) for the determina-

tion of arginine. Bound taurocyamine was estimated by the same method after hydrolysis of the N-P bond in *N*-HCl at 100° for 10 min.

Inorganic phosphate was estimated by the method described by Ennor & Stocken (1950).

All hydrolyses and enzyme experiments were carried out with the sodium salts of monophosphotaurocyamine and fraction II (see later); these were prepared by passage of solutions of the barium salts through columns of Zeo-Karb 225 (Na⁺ form).

Myosin was prepared by Dr H. Rosenberg of this Department from rabbit skeletal muscle as described by Bailey (1942). It was stored in 50% (v/v) glycerol at -10° and contained 2.5 mg. of protein/ml.

Taurocyamine phosphokinase (TPK) was purified from aqueous extracts of *Arenicola assimilis* by a method which will be described later and which involved fractionation with acetone, ammonium sulphate and ethanol. The enzyme was stable and was stored in 50% (v/v) glycerol at -10°; the solution contained 1.86 mg. of protein/ml.

The purified intestinal alkaline phosphatase was a gift from Professor R. K. Morton, University of Adelaide.

Taurocyamine was prepared by allowing *S*-methylisothiouraea to react with taurine in an alkaline medium in the technique described by Schutte (1943) for the preparation of guanidines. Thus 72 g. of taurine was dissolved in 250 ml. of aq. 17*N*-NH₃ soln. and 120 g. of *S*-methylisothiouraea sulphate added. The mixture was heated to 60° and stirred vigorously until the reactants dissolved. There was a vigorous evolution of methanethiol and the mixture was allowed to cool to room temperature. Crystallization of taurocyamine commenced after a few minutes and the reaction was allowed to proceed for 20 hr. The crystalline material was filtered off, washed with ice-cold water and

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the filtrate discarded. The solid material was recrystallized several times from water. Yield, 50 g.; m.p. 265° [cf. m.p. 228–230° (van Thoi & Robin, 1954); m.p. 264° (Dittrich, 1878); m.p. 228° (Engel, 1875)]. The purity of the material was checked by N analysis and paper chromatography.

RESULTS

Initial experiments. Taurocyamine was allowed to react with POCl_3 in an alkaline medium and the barium salt of the product isolated from the mixture. This salt was assumed to be the monophosphate, i.e. $\text{C}_3\text{H}_8\text{O}_6\text{N}_3\text{SPBa}$, but analysis of the material dried over P_2O_5 *in vacuo* at 100° showed Ba = 40.8% (theory = 35.8%) and P:taurocyamine ratio = 1.10. Two samples prepared subsequently gave P:taurocyamine ratios 1.33 and 1.44, indicating contamination by a compound containing P. Determinations were made of P and taurocyamine released from the compound during hydrolysis in N-HCl at 37°. There was a progressive decrease with time of the P:taurocyamine ratio from a maximum value of 1.81 (Table 1), which was interpreted as indicating the presence of a mono- and di-phosphotaurocyamine. As a consequence some of the material was placed on an anion-exchange

resin (IRB-400, Cl^- form), from which two fractions were subsequently eluted: one with 0.1M-NaCl having a P:taurocyamine ratio = 1:1, the other with 2M-NaCl and a ratio = 2:1 (fraction II). The details of the synthesis and separation of the two phosphorylated derivatives are given below.

Preparation of crude phosphotaurocyamine. Taurocyamine (20 g., 0.12 mole) was added to a three-necked flask (2 l.) containing 20 ml. of water and 50 ml. of 10N-NaOH. The mixture was stirred rapidly and 38 ml. (0.43 mole) of POCl_3 and 170 ml. of 10N-NaOH were added over a period of 1 hr. and at such a rate that the mixture remained strongly alkaline. The contents of the flask were maintained between -5° and 0° by periodic immersion in a solid CO_2 -ethanol mixture. When the additions were completed the reaction mixture was stirred for a further 30 min. at -5° . The precipitate of NaCl and inorganic P was removed by filtration on a Büchner funnel (Whatman no. 531 paper) and the solid residue extracted with 200 ml. of ice-cold water in a Waring Blender. The mixture was filtered and the residue re-extracted twice with 100 ml. of water. The residue was discarded; the four filtrates were combined and allowed to stand for 24 hr. at 5° . The crystalline precipitate which formed was removed by filtration and discarded, and the filtrate was adjusted to pH 7.5 by the addition of 5N-HCl. Ethanol (4 vol.) was then added and the oil which separated allowed to settle for 24 hr. at 5° . The supernatant liquid was discarded after removal by decantation and the semi-solid oil dissolved in 500 ml. of water. This solution was clarified by filtration and the sodium salts were again precipitated as an oil by the addition of ethanol (4 vol.). This mixture was allowed to stand for 24 hr. at 5° , the supernatant decanted and the oil dissolved in 500 ml. of water and filtered. These two precipitations removed the free taurocyamine and a large proportion of the inorganic P (Table 2).

Isolation of monophosphotaurocyamine. The aqueous solution of the twice-precipitated oil was

Table 1. *Hydrolysis of crude phosphotaurocyamine*

Crude phosphotaurocyamine (1.1 mM on the basis of taurocyamine) was hydrolysed in N-HCl at 37°.

Time (min.)	P ($\mu\text{moles/ml.}$)	Taurocyamine ($\mu\text{moles/ml.}$)	Molar ratio P:Taurocyamine
2.5	0.047	0.0215	1.81
5	0.075	0.038	1.75
10	0.127	0.07	1.69
40	0.406	0.250	1.59
50	0.458	0.302	1.48
120	0.845	0.588	1.42
180	1.04	0.755	1.36
	1.47*	1.09*	1.34*

* These results were obtained from a sample hydrolysed in N-HCl at 100° for 15 min.

Table 2. *Analytical data on fractions obtained in the isolation of barium salts of monophosphotaurocyamine and fraction II*

Fraction	Vol. (ml.)	Taurocyamine (g.)			Ba salt (g.)
		Free	Total	Bound	
Initial filtrate	1220	6.2	15.3	9.1	—
Soln. of oil (first ppt.)	500	0.3	8.3	8.0	—
Soln. of oil (second ppt.)	500	0.03	7.6	7.6	—
Fraction 0	960	0.03	0.7	0.7	0.9
Monophosphotaurocyamine	2980	0.01	4.7	4.7	10.8 (*7.0)
Fraction II	540	0.003	2.2	2.2	9.7 (*5.5)

* These figures represent the final yields after two further precipitations in the absence of added Ba acetate. This procedure results in appreciable losses but is necessary if serious contamination of the product with barium acetate is to be avoided.

passed through a column (66 cm. $\times$ 3 cm.) of De-Acidite FF (Cl^- form) at approx. 5 ml./min. and the effluent tested at intervals for the presence of bound taurocyamine. In all preparations it was found that some bound guanidine (fraction 0) passed through the column to the extent of 1–10% of the total bound guanidine present in the original solution. Chromatographic examination of this material after hydrolysis and development on paper in methanol–acetic acid–water (80:10:20, by vol.), *n*-butanol–6*N*-HCl (70:30, v/v) (Makisumi, 1952) and phenol–water (80:20, w/v) saturated with SO_2 (Crawford, 1951) indicated the presence of two guanidines. One of these was identical in behaviour with taurocyamine but the other, which had a higher R_f in all solvents, was not identified. This material was discarded. The column was then washed with water until the effluent was free of bound taurocyamine. Elution was then commenced with 0.2*M*-NaCl (5 ml./min.) and the eluate collected in 200 ml. fractions. Qualitative tests for bound taurocyamine were carried out on each fraction and fractions 1–15 were combined. Elution with additional 0.2*M*-NaCl removed only trace amounts of bound taurocyamine. Barium acetate (75 ml. of *m*-solution) was then added to the combined eluates and the pH adjusted to 8.2 by the addition of *N*-NaOH. The precipitate was removed by filtration and the filtrate adjusted to pH 4–5 by the addition of 5*N*-HCl. The solution was then placed in a flask, which was evacuated on a water-pump to remove CO_2 , and the solution brought to pH 6–6.5 by the addition of a freshly prepared solution of NaOH. Ethanol (3 vol.) was added and the amorphous precipitate collected by centrifuging, washed with ethanol and ether and dried *in vacuo* over CaCl_2 . The dry material was dissolved in 250 ml. of water, adjusted to pH 6 and precipitated by the addition of ethanol (2 vol.) in the absence of added barium acetate. The precipitate was collected as before, dried and again similarly precipitated. Yield, 7 g. (Table 2).

The material on analysis gave C, 8.8; H, 2.8; N, 9.6; S, 7.1; P, 7.3; Ba, 31.6; taurocyamine, 39.0; H_2O , 8.7; ash ($\text{Ba}_3\text{P}_2\text{O}_7$), 52.5. Calc. for $\text{C}_3\text{H}_5\text{O}_6\text{N}_3\text{SPBa}_2\text{H}_2\text{O}$: C, 8.6; H, 2.9; N, 10.0; S, 7.6; P, 7.4; Ba, 32.7; taurocyamine, 40.0; H_2O , 8.6; ash, 53.8%. The compound is believed to possess the structure:

Attempts to isolate the compound as the crystalline sodium, potassium and lithium salts were unsuccessful because of their markedly hygroscopic nature, but the crystalline ammonium salt was prepared. The barium salt (400 mg.) was dissolved in 20 ml. of water and passed through a column (1 cm. $\times$ 25 cm.) of Zeo-Karb 225 (NH_4^+ form). The

column was washed with water and the washings (30 ml.) were combined with the original effluent. Ethanol (6 vol.) and acetone (6 vol.) were then added and the finely dispersed oil which separated crystallized in fine needles on standing at 5° overnight. The material, on analysis, corresponded to the empirical formula $\text{C}_3\text{H}_5\text{O}_6\text{N}_3\text{SP}(\text{NH}_4)_2$.

Isolation of fraction II. After elution of monophosphataurocyamine the column was treated with 2*M*-NaCl to elute fraction II, which was collected in 540 ml. of eluate. Barium acetate (50 ml. of *m*-solution) was added and the solution became slightly opalescent but cleared when adjusted to pH 4–5 by the addition of 5*N*-HCl. Dissolved CO_2 was removed as before and the pH brought to 7. (At higher pH values a precipitate appears and this increases with increasing alkalinity.) Ethanol (2 vol.) was then added and the pH re-adjusted to 7 by the addition of 5*N*-NaOH. The precipitated barium salt was recovered by centrifuging, washed with ethanol and ether and dried *in vacuo* over CaCl_2 . The dry material was dissolved in water and precipitated in the absence of additional barium acetate with ethanol (2 vol.). The precipitate, after drying, was redissolved, similarly reprecipitated and again dried. Yield, 5.5 g. (Table 2).

Fraction II gave on preliminary analysis a Ba:P:taurocyamine ratio of 2:2:1, and it was anticipated that the compound would be diphosphataurocyamine and that its structural formula could be expressed thus:

Full elementary analysis, however, revealed the presence of an excess of C, H and S over that demanded by theory on the basis of the above formulation. It seemed likely that the excess of these elements might be due to a contaminant eluted from the column by the 2*M*-NaCl. To test this supposition some of the material was dissolved and the solution passed through another column of De-Acidite FF (Cl^- form). The column was washed with water and then with 0.1*M*-NaCl until appreciable amounts of bound taurocyamine appeared. The remainder of fraction II was then eluted with 0.4*M*-NaCl and the product isolated as a barium salt in the manner described above. On analysis the material gave figures similar to those mentioned above. Attempts made to crystallize the compound as an ammonium, potassium or sodium salt were unsuccessful. The questions whether fraction II was a diphosphoguanidine and whether taurocyamine was in fact the guanidine released under mild conditions of hydrolysis were checked by the chromatographic behaviour of the guanidine released by hydrolysis in 0.1*N*-HCl at 65°; the material was chromatographed (Whatman no. 3MM) in a variety of solvent systems and in all

cases behaved similarly to authentic taurocyamine. The solvent systems used were acetone-aq. 2*N*-NH₃ soln.-water (60:30:10), propanol-aq. NH₃ soln. (sp.gr. 0.88)-water (60:30:10), Hanes & Isherwood (1949), and pyridine-*isopentanol*-water (80:40:70), Roche, van Thoi & Hatt (1954).

Stability in acid. The relative stabilities of monophosphataurocyamine and fraction II were determined by hydrolysis in *N*-HCl at 37°. With the monophosphate, P and taurocyamine were released in equimolar proportions throughout the course of the hydrolysis. The compound was 50% hydrolysed in about 70 min.

The behaviour of fraction II under similar conditions presented a marked contrast. Thus, owing to a difference in the stabilities of the phosphoryl linkages, the P:taurocyamine ratio in the early stages of the hydrolysis was about 4:1 and fell as hydrolysis proceeded. Fraction II was 50% hydrolysed in about 130 min., at which time the P:taurocyamine ratio was about 2.3:1.

Stability in alkali. No taurocyamine was released from either the monophosphate or fraction II when heated in *N*-NaOH for 60 min. at 37°.

Action of phosphatase. In an attempt to obtain some idea of the nature of the phosphoryl linkages in fraction II and to compare its behaviour with that of the monophosphate both were incubated with alkaline phosphatase. The results indicated that P and taurocyamine are released from the monophosphate in equimolar proportions throughout the course of the reaction and that the compound is completely hydrolysed by the enzyme. With fraction II (Fig. 1), on the other hand, the rate of liberation of P was much greater than that of taurocyamine. Thus at the end of 5 min. the molar ratio P:taurocyamine was 18 and after 20 min. had fallen to 5. After 75 min. both P and taurocyamine were being released at a slow constant rate with a P:taurocyamine ratio of approx. 2.6. Fraction II was hydrolysed only to the extent of about 55% after 240 min.

Taurocyamine phosphokinase experiments. The

ability of fraction II to function as a substrate for the enzyme taurocyamine phosphokinase (TPK), which catalyses the reaction

has been tested in the presence and absence of myosin. This latter enzyme was added to ensure that the maximum amount of taurocyamine would be released. Similar experiments carried out as controls, with the monophosphate as substrate, indicated (Table 3) that at equilibrium 55% of the taurocyamine was released. In the presence of myosin all bound taurocyamine was released. Such a result would, of course, be expected as the monophosphate is the natural substrate for the enzyme. The results with fraction II indicate that only a small proportion of bound taurocyamine is released even in the presence of myosin. It is of interest to note that the ratio of the taurocyamine present at equilibrium to that present when myosin was added is the same for both the monophosphate and fraction II.

Fig. 1. Action of alkaline phosphatase on fraction II. The reaction mixtures contained ethanolamine (0.05*M*, pH 9.2); alkaline phosphatase (18 μg. of protein/ml.); fraction II (0.98 *mM* with respect to taurocyamine). Temp. 37°. O, Taurocyamine; Δ, P released.

Table 3. Monophosphataurocyamine and fraction II as substrates for taurocyaminephosphokinase

Reaction mixture: adenosine diphosphate (5*mM*); Mg²⁺ ions (2.5 *mM*); Ca²⁺ ions (5 *mM*); *N*-ethylmorpholine (0.05*M*, pH 7.2); TPK (50 μg. of protein/ml.); monophosphataurocyamine (2.67 *mM*) or fraction II (1.33 *mM*, calculated on bound taurocyamine content). Total volume, 1.5 ml. Reaction time, 8 hr. at 37°.

	Monophosphataurocyamine		Fraction II	
	No myosin (a)	Myosin (800 μg. of protein/ml.) (b)	No myosin (a)	Myosin (800 μg. of protein/ml.) (b)
Taurocyamine (μmoles/ml.)	1.48	2.67	0.16	0.28
Taurocyamine (% released)	55	100	12	21
a/b	0.55		0.57	

DISCUSSION

The reaction between phosphoryl chloride and glycoeyamine or creatine to yield *N*-phosphorylated derivatives was initially described by Zeile & Fawaz (1938) and subsequently by Ennor & Stocken (1948), who isolated phosphocreatine as the sodium salt. These communications did not refer to any side reactions and only one product was isolated. It was therefore anticipated that the phosphorylation of taurocyamine with phosphoryl chloride under similar conditions would yield but one product, i.e. a monophosphate. Such, however, was not the case and the separation of this latter compound from a mixture could be accomplished only by the use of an anion-exchange resin, from which it was eluted by 0.2M-NaCl. Although the compound could be crystallized as the ammonium salt it was isolated as a pure amorphous barium salt.

Experiments with acid hydrolysis indicated the usual lability of the N-P bond, but it is of some interest to note that the compound is much more stable in acid than is phosphocreatine. Thus this latter substance is 50% hydrolysed in 0.1N-HCl at 37° in 15 min. and is completely hydrolysed in 90 min., whereas monophosphotaurocyamine is 50% hydrolysed in N-HCl at 37° in 90 min. In the presence of acid molybdate phosphocreatine is more rapidly hydrolysed than in acid alone, but acid molybdate has no effect on the rate of hydrolysis of phosphotaurocyamine.

This lack of influence of molybdate on the hydrolysis of the monophosphate is of interest in view of the results of Baldwin & Yudkin (1950). These authors found that the rate of hydrolysis of the phosphagens of certain annelids and gephyreans in acid was retarded in the presence of molybdate. Moreover the 'retardation factor' was smaller than could be explained on the basis of the presence of phosphoarginine, the hydrolysis of which is markedly inhibited by molybdate. The monophosphate is completely hydrolysed by alkaline phosphatase, which is believed to attack only N-P and ester-phosphate linkages (Morton, 1955). In the dry form as the barium salt it is unstable and slowly breaks down over 6 months at room temperature to yield the free base and inorganic P. It has, however, been found that the compound is stable in solution as the sodium salt if stored at -15°; such solutions have been kept for 6 months without decomposition.

The other product of the phosphorylation is readily separated from the monophosphate but is of unknown constitution except insofar as it yields, on acid hydrolysis, 2 moles of P to 1 mole of taurocyamine. There is, however, no evidence as to the manner in which the P is bound, although it is

clear from the phosphatase experiments that a part of the P is bound in an N-P linkage. If it be assumed that monophosphotaurocyamine is the natural substrate for TPK, and that the enzyme has a high degree of specificity for this substrate, additional evidence as to the nature of fraction II can be obtained from the action of this enzyme upon the monophosphate and fraction II. The fact that the ratio of the taurocyamine released in the absence and in the presence of myosin is about the same with monophosphotaurocyamine and fraction II as substrates is indicative of a contamination of this latter with the monophosphate. Thus it may be calculated that fraction II is contaminated to the extent of 20% with monophosphotaurocyamine. It is, however, possible that such a contamination arises as a result of breakdown of the original 'compound' as first isolated from the ion-exchange resin. This is perhaps likely, particularly as fraction II when isolated as the barium salt breaks down in such a way that 50% of the bound taurocyamine appears as the free guanidine even when stored in the dry state at room temperature (24°) over 4 months.

Just before submission of this paper for publication the report of the details of the synthesis and isolation of the ammonium salt of phosphotaurocyamine by van Thoi & van Thiem (1957*b*) came to hand. Phosphorylation of taurocyamine, as in the present work, was carried out with phosphoryl chloride but under different conditions, and there was no evidence for the formation of a phosphorylated product other than monophosphotaurocyamine. The isolation procedure involved the handling of large volumes and is thus less convenient than the method described here.

SUMMARY

1. A method is described for the preparation of the barium salt of *N*-phosphoguanidinoethanesulphonic acid.
2. A phosphorylated product in addition to the above is obtained. This can be readily separated from the reaction mixture but is of unknown constitution.
3. Some of the properties of *N*-phosphoguanidinoethanesulphonic acid are described.

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Biosynthesis of Intestinal Mucins

2. INCORPORATION OF [³⁵S]SULPHATE BY GUINEA-PIG COLON *IN VITRO**

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In a previous paper (Pasternak, Kent & Davies, 1958) the uptake of [³⁵S]sulphate by isolated gastro-intestinal tissues of a number of animals was surveyed; the highest incorporation was found to occur in guinea-pig colon. This tissue was accordingly chosen for a more detailed investigation of fixation of [³⁵S]sulphate. A brief account of part of this work has already been presented (Pasternak, Kent & Davies, 1956).

METHODS

Preparation of tissue. Tissue was prepared as described by Pasternak *et al.* (1958).

Incubation. The incubation medium was that described by Pasternak *et al.* (1958), glucose being omitted. Substrates were added to yield final concentrations of 0.02M, and incubated with tissue for 3 hr. except where otherwise stated; sodium pyruvate was kindly given by Mr R. W. Wakelin. Other details of incubation were as described by Pasternak *et al.* (1958).

Extraction of tissues. Tissues after incubation were extracted with 5 ml. of NaOH (2%, w/v) for 12 hr. at room temperature. The resulting extracts were centrifuged to remove residual cell fragments before dialysis. This treatment did not result in significant loss of bound sulphate by hydrolysis.

Dialysis. Dialysis of extruded mucin (i.e. that which passes into the incubation medium) and extracted mucin (i.e. mucin obtained from the tissue after incubation) (as defined by Pasternak *et al.* 1958) was carried out in cellophan against running tap-water (16°), constant activity being reached within 90 hr. Carrier Na₂SO₄ and penicillin plus streptomycin were added every 12 hr. as described by Pasternak *et al.* (1958).

Electrophoresis. The method of Kunkel & Tiselius (1951) was employed, sodium diethylbarbiturate buffer (0.07M, pH 8.8) and Whatman no. 1 paper (8 cm. × 32 cm.) being used. Three mucin samples (0.025–0.1 ml.) were investigated simultaneously; by using three paper strips and four glass plates 'sandwich-wise' up to nine samples could be included in one run. At the end of electrophoresis (6 hr., 120V; 4.3V/cm.) the paper was dried and cut into longitudinal strips, each strip containing the separated components of one sample.

Dry-weight determination. Dry weights of tissues were determined as described by Pasternak *et al.* (1958).

Radioactive assay. Samples of the mucin solutions before and after dialysis were assayed as described by Pasternak *et al.* (1958). Alternatively, the proportion of added isotope in the [³⁵S]mucin was obtained by paper electrophoresis of a sample (0.05–0.08 ml.) of the medium or tissue extract after incubations.

Paper strips after electrophoresis were assayed (GM mica end-window tube EHM2s operating at 1640V) as contiguous rectangles (1.7 cm. × 1 cm.) along the length of the strip. The radioactive peak at the origin was identified as [³⁵S]mucin, whereas that which had migrated towards the anode was shown to be free sulphate (approx. 1.4×10^{-2} cm.²/hr.). The term [³⁵S]mucin is used throughout this paper to denote ³⁵S-labelled, [³⁵S]sulphate-free extruded or extracted mucin. It was considered unnecessary to correct for self-absorption, since the thickness of the paper, which is decisive for this isotope, is the same for both radioactive components. The radioactivities of the two components were determined by summing the counts below each of the plotted peaks and correcting for background (approx. 10 counts/min./cm.). The proportion of added isotope found in the [³⁵S]mucin was calculated from the ratio $\{[\text{³⁵S]mucin (counts/min.)} \times 10^5\} : \{[\text{³⁵S]sulphate} + [\text{³⁵S]mucin (counts/min.)}\}$ and expressed as counts/min./100 000 counts/min. initially added/mg. of tissue dry wt. (subsequently abbreviated as counts/mg. of tissue). [³⁵S]Mucin (counts/min.) represents the total counts given

* Part 1: Pasternak, Kent & Davies (1958).