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Studies on Tissue Distribution, Fluorescent Properties, and Iodination of the Plant Alkaloid, Berberine*

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Introduction

At present, the X-ray diagnosis of pancreatic disease can be accomplished in three general ways. First, the pancreatic ducts can be injected with radiopaque media during operative exposure, [1,2] secondly, the changes in some other organ produced by a lesion of the pancreas may be observed (e.g. displacement of the stomach by a cyst) and, finally, direct radiographic visualization is possible when pancreatic calcification has occurred. If to these rather limited diagnostic measures there could be added a safe, non-operative means of X-ray visualization of the entire pancreas, the clinician would be equipped with a very useful tool for the early detection of pancreatic disorders such as tumours.

To this end it would be most desirable to find or to produce a radiopaque substance having selective affinity for the pancreas. Among the materials which are known to be eliminated by the pancreas following parenteral administration are various organic dyes,[3,4] sulphadiazines,[5] a few antibiotics,[5] and the metals zinc and manganese.[6] None of these substances are normally radiopaque, and various attempts[6–8] to prepare derivatives with this property have resulted in compounds which are too insoluble, too toxic, or insufficiently concentrated in the pancreas to produce

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radiopacification. Recently, McCune and Stanbro[9] have used trypsin to alter the ability of the pancreas to absorb contrast media, and diffuse radiopacification of pancreatic tissue in dogs was apparent on X-ray examination after intra-arterial injection. However, this technique is not currently applicable to human beings.

In 1955, a programme[10] for the screening of various dyes was begun, following several reports[3,4] on the appearance of injected dyes in pancreatic juice. It was of considerable interest, therefore, when a communication by Nardi and Seipel[11] revealed quantitative evidence that the plant alkaloid, berberine, was selectively localized in pancreatic tissue of rats. From subcutaneously injected doses of 75 mg of berberine chloride, recoveries as high as 70 per cent of the berberine were reported from a few rat pancreata. Visual evidence for this pancreatic selectivity had been pointed out previously by Taylor,[12] who observed a bright yellow fluorescence under ultraviolet light in pancreatic tissue of mice following injection of extracts from the columbo root.[13,14] This plant (*Jateorhiza palmata* Lam., Miers; *J. columba* Miers) contains a mixture of alkaloids closely related in structure and properties to berberine and showing, like berberine, a characteristic yellow fluorescence under ultraviolet light. Nardi and Seipel used berberine* in their later work when they found that it also produces pancreatic fluorescence in both rats and mice.

On the basis of these reports we investigated the preparation of iodinated derivatives of berberine and related alkaloids as agents for pancreatic radiography. One iodinated compound with the berberine structure, 9-demethyl-9-*p*-iodobenzylberberine chloride (IX, see part III), was prepared and found to produce pancreatic fluorescence but no radiopacification.

During the course of this work we received a communication from Blau[15] reporting that he had been unable to obtain any evidence of the selective localization of berberine in pancreatic tissue of injected rats by use of spectrophotometric methods of analysis. This work by Blau and Bender[16] has been published recently in greater detail. Since their conclusions conflicted with those reported previously,[11] it was necessary for us to repeat the

* The pharmacological properties of berberine have been reviewed by Shideman.[27]

work of Nardi and Seipel, who supplied all necessary experimental details.[17,18] In our hands their procedure for determining berberine gravimetrically from pancreatic tissue yielded inconclusive results which indicated, however, that no large quantities of berberine were present in the pancreas of injected rats. For confirmation we have developed other, more precise, analytical methods for determining berberine in animal tissue including gravimetric, spectrophotometric, and radio-isotope assay procedures.

Our results confirm the findings of Blau and Bender. Small amounts of berberine were identified after injection under many different conditions in a number of rat tissues, of which the total liver consistently contained the largest quantity, about two to six times the amount in the total pancreas. The maximum quantity of berberine identified in any single rat pancreas after injection of at least 10 mg was about 0·18 mg, determined by radio-isotope assay.

In this paper we report our methods of analysis and their results, our observations of the fluorescent behaviour of berberine and related compounds in animal tissues, and some of our work on the iodination of berberine, including the preparation of 9-demethyl-9-*p*-iodobenzylberberine chloride.

I. Analysis of Berberine in Rat Tissues

A. Gravimetric Methods of Analysis

1. *Procedure of Nardi and Seipel*

The initial work of analysing for berberine was directed towards repeating the work of Nardi and Seipel.[11] They had utilized the picrolonic acid precipitation method of Richter[19] to determine berberine in pancreatic tissue extracts of injected rats. Their procedure[17,18] may be summarized as follows:

White rats, male and female, weighing about 240 g, were lightly anaesthetized with ether and injected subcutaneously between the 'shoulder blades' with 75 mg of berberine chloride in propylene glycol solution; the localized swelling was spread by brief massage over the back. This dose was lethal in 4–6 h for almost all animals. Each pancreas, easily recognized by the bright yellow fluorescence of berberine, was dissected out, weighed, and dried at 85° for 12 h. The tissue was loosened from the glass, treated

with 5 ml of 95 per cent ethanol containing 5 drops of concentrated hydrochloric acid, dried at 85° for 12 h, and reweighed. After three extractions with 30 ml of petroleum ether the material was dried for another 12 h and broken up to a fine powder. The dry, defatted pancreas was boiled gently with 20 ml of 95 per cent ethanol for 5 min and filtered through glass wool. The residue was extracted once with an additional 10 ml of boiling ethanol, once with 6 ml of boiling 2 N hydrochloric acid, and was permitted to stand overnight with 5 ml of ethanol. The combined filtrates were reduced to a volume of about 15 ml with evaporation of all the ethanol and addition of water, if necessary, and then extracted twice with 30 ml of petroleum ether.

The solution was made barely alkaline to phenolphthalein, and an excess of saturated alcoholic solution of picrolonic acid was added. The precipitate was collected on a tared fritted glass crucible, washed with water until the washings were colourless, then with 50 per cent absolute alcohol–ether, and finally with ether. The precipitates were weighed after drying at 55° for one hour.

Using this procedure Nardi and Seipel reported yields of berberine picrolonate from the pancreata of 22 rats ranging from 71·6 per cent to 1·0 per cent (median around 31 per cent) of the injected dose.

In our hands this procedure* gave no significant quantities of precipitate from pancreatic tissue of injected rats. Our results are summarized in Table I. The infrared spectrum of each precipitate was recorded and compared with that of pure berberine picrolonate. The samples from the control pancreata were essentially identical in their spectra to those from the pancreata of rats injected with berberine, and none of the latter showed any detectable quantities of berberine picrolonate. Samples to which berberine chloride was added originally showed the infrared spectrum of berberine picrolonate plus varying amounts of impurities.

2. *Acid digestion of tissues*

In an attempt to improve the method, the tissues were digested with acid before precipitation with picrolonic acid. Acid rather than base was used as the digestion medium since the berberine ring system tends to be unstable in the presence of excess alkali.[20] Rat pancreata as well as spleens, salivary glands, and livers were cut into small pieces and digested with warm concentrated

* Our injections were usually made in the subcutaneous tissue of the abdominal wall rather than between the scapulae.

hydrochloric acid for 3 to 7 days until no further breakdown of tissue was apparent. Residues were evaporated and extracted with 95 per cent ethanol which was then filtered through sintered glass or glass wool. The ethanol was evaporated under nitrogen and replaced by 15 ml of water before addition of picrolonic acid.

All tissue except liver responded well to this treatment which, in addition, was found to have little or no effect on pure berberine

Table I. Determination of berberine by the method of Nardi and Seipel[11, 17, 18]

Conditions Tissue	Berberine.Cl.$2H_2O$, mg added before digestion	Precipitate found, mg	Recovery as berberine picrolonate, %
None	49·5	59·0	81
None	6·6	4·4	45
Pancreas from control rats	none	0	—
	none	2·0	—
	none	1·7	—
	none	1·0	—
Pancreas from control rats	49·1	37·5	52
	15·6	8·7	38
	6·3	6·6	71
	2·3	0·6	18
Pancreas { from rats injected subcutaneously with 75 mg/kg (~17 mg/rat) of berberine.Cl.$2H_2O$ sacrificed after 5 h		1·1	—
		0·4	—
		1·7	—
		2·7	—
Pancreas { from rats injected subcutaneously with 75 mg/rat of berberine.Cl.$2H_2O$, sacrificed after 4·5 h		1·8	—
		3·8	—
		2·0	—

chloride. However, the acid degradation liberated large quantities of basic compounds capable of forming stable precipitates with picrolonic acid. These impurities, even in very small quantities, cause berberine picrolonate to form a very fine slimy precipitate which is extremely difficult to filter or to purify.

3. *Determination of berberine as berberine acetonate*

To avoid these problems, we investigated a gravimetric method[21] more specific to berberine using acetone, which forms an extremely water-insoluble addition product with berberine and related alkaloids in dilute base. This procedure was found to be very

satisfactory for determining pure berberine in quantities of from 10 to 50 mg and for detecting at least 0·6 mg of pure berberine. In the presence of the tissue extracts, varying amounts of interfering substances were obtained. These could have been removed by chromatographic purification of the solutions as described below. However, further attempts to improve this procedure were abandoned because it became apparent that the quantities of berberine present in the pancreata of injected rats were far too small (less than 0·5 mg) for gravimetric analysis.

B. Spectrophotometric Method of Analysis

Small amounts of berberine can be determined accurately by spectrophotometric methods since this compound has a very strong absorption band at 340 to 345 mμ ($\epsilon = 28{,}100$). Blau and Bender[16] used a similar method in attempting to confirm the results of Nardi and Seipel.

1. *Chromatographic purification of tissue extracts*

Chromatography on activated alumina was found to be an excellent method for obtaining the berberine from the tissue extracts in sufficient purity for spectrophotometric analysis. Since berberine tends to be unstable under alkaline conditions,[20] the alumina (Fisher Adsorption Alumina, 80–200 mesh) was first treated with an equal weight of 1·5 per cent hydrochloric acid, washed three times with water, and dried for about 24 h at 200°. Berberine chloride is immediately and completely adsorbed on the alumina from chloroform solution, but it can be removed quantitatively by absolute ethanol or by ethanol–chloroform mixtures containing at least 20 per cent ethanol by volume.

Tissue extracts of rat pancreas, spleen, fascia, salivary gland, brain, or kidney were prepared, usually by acid degradation as described above. Rat liver extracts were prepared by cutting the tissue into small pieces and extracting with 95 per cent ethanol until no more yellow fluorescence appeared in fresh ethanol after a minimum of 24 h. These ethanol solutions as well as those from acid-degraded tissues were prepared for chromatography by filtering through sintered glass or glass wool, evaporating the 95 per cent ethanol under nitrogen, and replacing it by

absolute ethanol (50 to 100 ml as required). Originally it had been thought necessary to purify the residues further before chromatography by dissolving them in water, filtering, extracting with petroleum ether, and re-evaporating in preparation for dissolving them in absolute ethanol as above. This procedure was used to obtain the results in Tables III and IV. However, losses in berberine did occur (Table III) due, largely, to excess handling. Later it was found (results in Table V) that equivalent purification was obtained without loss of berberine by omitting the preparation of water solutions and the petroleum ether extractions.

The absolute ethanol solutions of the tissue extracts were first purified crudely by passage through a large column containing 50 to 100 g of alumina packed with glass wool. (The use of sea sand in these columns was eliminated when one sample was found to adsorb berberine irreversibly.) Elution with absolute ethanol caused any berberine present to pass rapidly through the column as a brilliant yellow fluorescent band while most of the impurities were retained tenaciously by the alumina. For further purification, the eluate was evaporated under nitrogen to a small volume (1 to 3 ml) and treated with 30 to 100 ml of ethanol-free chloroform to make a solution containing no more than 2 to 4 per cent ethanol. This was placed on a small column prepared in a burette with 25 g of alumina. After washing the column with chloroform to remove an oily yellow impurity band, the berberine was eluted with 20 per cent ethanol in chloroform. This solvent was evaporated to dryness, and the berberine was dissolved in water and placed in a volumetric flask of appropriate size to give some concentration between 1×10^{-5} and 3×10^{-5} M if possible.

2. *Spectrophotometric determination of berberine*

The instrument used for this work was a Beckman Model DU Spectrophotometer. The sensitivity was always set so that a slit opening of about 0·65 mm was obtained at 320 mμ, giving about 0·29 mm slit width at 345 mμ with the solvent cell (distilled water). Berberine concentrations in the chromatographed tissue extracts were determined by a background cancellation method, since all samples showed various amounts of background absorption. The absorption at 345 mμ was determined by drawing a line between the optical density values at 330 mμ and 360 mμ and

measuring the peak height from the centre (345 mμ) of this line. When this peak height was plotted against concentration for a series of standard solutions from $0 \cdot 5$ to 3×10^{-5} M berberine chloride, a straight line going through the origin was obtained. Known amounts of berberine were then added to control tissue extract solutions, and their concentrations, determined by using this graph, agreed well with the known values, as shown in Table II.

Table II. Berberine chloride added to control tissue extracts

Added (molarity)	Found (molarity)
None	$-0 \cdot 01 \times 10^{-5}$
$0 \cdot 05 \times 10^{-5}$	$0 \cdot 03 \times 10^{-5}$
$0 \cdot 10 \times 10^{-5}$	$0 \cdot 09 \times 10^{-5}$
$0 \cdot 20 \times 10^{-5}$	$0 \cdot 19 \times 10^{-5}$

Since the background absorption curves have somewhat concave shapes in the region between 330 and 360 mμ, the concentrations determined are always somewhat lower than the true values. The magnitude of this error is roughly proportional to the optical density of the background absorption at 320 mμ. For this reason, the quantity of berberine determined by this procedure is reported as a range whose lower limit is the observed value and whose upper limit expresses the maximum probable spectrophotometric error. The smallest amount of berberine chloride tetrahydrate that one could measure quantitatively by this means in any one tissue extract was $0 \cdot 002$ mg.

The entire analytical method was tested by treating known amounts ($0 \cdot 016$–$0 \cdot 040$ mg) of pure berberine chloride with warm concentrated hydrochloric acid for several days and then carrying the samples through the chromatographic procedures and the spectrophotometric determinations. In each case, 95 to 100 per cent of the berberine was recovered. Losses did occur (Table III) however, when berberine chloride was added to control tissues which were carried through the above procedures including, in addition, the preparation of water solutions and petroleum ether extractions. These losses were due undoubtedly to excess handling and would affect the results in Tables III and IV. However,

Table III. Series I: Spectrophotometric determination of berberine in animal tissue. Milligrams of berberine.Cl.$4H_2O$[a]

Tissue	Controls	Berberine.Cl. $4H_2O$ added to excised tissue	Tissues from rats injected subcutaneously with berberine.Cl.$4H_2O$: Injected with 64 mg, sacrificed after 11 h	Injected with 67 mg, sacrificed after 4·5 h	Three rats each injected with 56 mg, sacrificed after 11 h	Rats injected between scapulae with 75 mg (exact procedure of Nardi and Seipel[11,17,18]): Rat No. 1: wt., 460 g. Died and autopsied after 3·5 h	Rat No. 2: wt., 358 g. Sacrificed after 4 h
Pancreas	0	Added: 10·0 Found: 6·7–6·8 (67–68%)	0·01–0·04	0·02–0·04	0·11–0·15 from 3 rat pancreata	0·07–0·08	0·06
Liver	0	Added: 10·0 Found: 5·8–5·9 (58–59%)	0·01–0·04	0	0·13–0·18 from 3 rat livers	0·18–0·19	0·14–0·21
Salivary glands		Added: 5·0 Found: 3·0–3·2 (60–64%)					
Spleen		Added: 25·0 Found: 20·9–21·3 (84–85%)				<0·002	0·003–0·005
Kidney						0·36–0·38[b]	<0·002
Brain						0·02–0·03	0·31–0·32[c]
Fascia				10·4–10·7			

[a] These results are probably somewhat low due to mechanical losses of berberine.

[b] High concentrations or crystals of berberine were frequently found in medullary areas of kidneys following berberine injections of all types, caused probably by low solubility of berberine in plasma leading to precipitation, lower nephron blockade, and renal failure.

[c] This large berberine concentration in the brain has not been explained. It was not seen in brain tissue from other injected rats (see Table VI).

Table IV. Series II: Berberine recoveries at varying times following intravenous injections. Milligrams of berberine.Cl.$4H_2O$, determined spectrophotometrically[a] from entire tissue

Tissue	1 min[b]	5 min[b]	85 min[b]	6 h[c]	12 h[c]	24 h[c]	36 h[c]	48 h[c]	72 h[c]
Pancreas	0·008–0·015	0·005–0·007	0·004–0·006	0·002	0·004–0·005	0·001–0·002	<0·002	0·001–0·002	<0·002
Liver	0·023–0·029	0·018–0·019	0·012–0·014	<0·002	<0·002	<0·002	<0·002	<0·002	<0·002
Salivary gland	<0·002	0	0·001–0·002	0	0	0	0	0	0
Spleen	0	0	0	0	0·001–0·003	0	0	0	0

[a] These results are probably somewhat low due to mechanical losses.

[b] These rats (female, 250–300 g) were injected in the femoral vein with 0·64 mg of berberine.Cl.$4H_2O$ as a 0·4 mg/ml solution in propylene glycol–saline. The animals died and were autopsied at the stated times after injection.

[c] These rats were injected as above except that less berberine (0·4 mg) was used and they survived until sacrificed at the stated times after injection.

these results are sufficiently reliable to demonstrate clearly any large concentration of berberine in the pancreas and to indicate the relative concentrations of berberine in various tissues.

3. *Results of spectrophotometric determinations*

(*a*) *Series I.* The first series of rats was injected subcutaneously, and various tissues were analysed, as described above, for berberine content. This series includes two rats that were injected and autopsied under conditions reproducing as closely as possible those of Nardi and Seipel. The results (Table III) indicate that roughly twice as much berberine can be found in the total liver as in the pancreas, where a maximum of 0·08 mg

Table V. Series III: Berberine recoveries at varying times following subcutaneous injections. Milligrams of berberine.Cl.$4H_2O$, determined spectrophotometrically from entire tissue

Tissue	5·5 h[a]	14 h[b]	24·5 h[c]	48·5 h[c]	125·5 h[c]
Pancreas	0·041–0·042	0·010–0·011	0·013–0·014	0·002–0·005	0
Liver	0·11 –0·12	0·070–0·076	0·028–0·035	<0·003	0·003–0·005

[a] This rat was injected with 56 mg of berberine in propylene glycol; it died and was autopsied 5·5 h later.
[b] This rat was injected as above, but it survived until sacrificed 14 h later.
[c] These rats were injected with less berberine (30 mg); they survived until sacrificed at the stated times.

of berberine was recovered by these procedures. Since the average rat liver weighs approximately ten times as much as the pancreas, the latter contains a greater concentration of berberine per gram of tissue than the liver. This slight degree of selectivity for the pancreas cannot be considered significant for purposes of pancreatic radiography because the concentrations involved are so small.

(*b*) *Series II.* A second series of rats was injected intravenously with berberine chloride and sacrificed at various time intervals to determine the rate of clearance of berberine and to detect any significant retention of berberine in the pancreas. The results (Table IV) show that the highest concentrations of berberine in most organs occur at the shortest time interval (one minute) after injection with no significant retention in any organ.

(*c*) *Series III.* Subcutaneous injections of berberine were made on a third series of rats, which were then sacrificed at various time intervals. These injections provided a reservoir of material, and any significant build-up of the berberine concentration in pancreas or liver could be detected. The results (Table V) again show highest concentrations at the shortest time intervals tested (5·5 h) after injection, with no indication of a build-up or retention of berberine in either organ.

C. Radio-isotope Method of Analysis

The third method of determining berberine in tissues of rats entailed the injection of radioactive berberine and the subsequent 'counting' of the excised organs. This procedure represents a very reliable check on the more detailed spectrophotometric results.

1. *Preparation of radioactive berberine*

Berberine acid sulphate was recrystallized from water and then sent to the New England Nuclear Assay Corp., Boston, Mass., to be tritiated by the method of Wilzbach.[22] A 5-g sample of the compound was sealed in an ampoule with 11 curies of tritium at 27°C and 0·27 atm for 7·2 weeks. A radiochromatogram of the tritiated product demonstrated that less than 50 per cent of the radioactivity was associated with the berberine molecule, the remainder presumably being with the water of hydration, the acid sulphate ion, and any impurities. To eliminate this extraneous radioactivity, the tritiated berberine acid sulphate was dissolved in hot water, filtered with activated charcoal, and converted to berberine (H^3) acetonate (H^1) by the procedure of Gordin.[21] The acetonate was washed with distilled water until the washings showed the presence of no more sulphate ion when tested with 5 per cent barium nitrate. The acetonate was then converted to berberine (H^3) chloride dihydrate (H^1) for use in the animal experiments. The acetonate dissolved in 45 ml of hot dilute (1:10) hydrochloric acid, and the chloride salt crystallized on cooling. The supernatant solution gave a negative test for sulphate ion. The berberine (H^3) chloride dihydrate (H^1) was obtained as fine yellow needles in 44 per cent yield after recrystal-

lization from water and drying over calcium chloride. The specific activity was determined to be 2·30 μc/mg. A radiochromatogram showed negligible extraneous radioactivity. A second sample was prepared from the same starting material by identical procedures except that it was not tritiated. Analysis showed that this was essentially pure berberine chloride dihydrate.

The decay of the tritium over the time interval used for the entire experiment was only 0·4 per cent. The loss of radioactivity due to exchange when the berberine (H^3) chloride dihydrate (H^1) was dissolved in water amounted to 15 per cent of the original activity over a 26-h period, and it appeared graphically to level off at that time.

2. *Injection procedures*

Four rats were used for these experiments. Two of them were injected subcutaneously with 60 mg (138 μc) of berberine (H^3) chloride dihydrate (H^1) dissolved in propylene glycol. There was evidence of some leakage at the sites of the injections. After 4·5 h the animals were sacrificed and the organs to be examined for radioactivity were removed immediately.

Two other rats were injected intravenously, one with 5 mg (11 μc) and the other with 10 mg (23 μc) of berberine (H^3) chloride dihydrate (H^1). These animals were sacrificed after sufficient time had elapsed to ensure circulation of berberine (about 2 to 5 min) and the organs were excised.

The pancreas, spleen, liver, salivary glands, lung, and brain of each of the four rats were placed in separate tubes and lyophylized. Of these organs, only the pancreas of each rat fluoresced bright yellow under ultraviolet light. The tubes were evacuated, sealed with hot Apiezon wax, and sent to the New England Nuclear Assay Corp. for counting.

3. *Results*

The results are given in detail in Table VI. In general they support the conclusions based on our earlier work. Berberine was found in small quantities in every organ tested. The pancreas showed the highest concentration of berberine per gram of tissue, but in most cases this was only of the order of twice the concentration in the liver or salivary glands and represented

Table VI. Radio-assay of organs of rats injected with berberine (H^3) chloride dihydrate (H^1)

(a) Rats Nos. 1 and 2 injected subcutaneously with 60 mg (138 μc)

Tissue	Specific activity, DPM H^3/mg tissue		Berb.(H^3).Cl. $2H_2O(H^1)$, μg/g tissue		Total dry tissue in average rat, g	Berb.(H^3).Cl. $2H_2O(H^1)$, mg/total average tissue	
	Rat No. 1	Rat No. 2	Rat No. 1	Rat No. 2		Rat No. 1	Rat No. 2
Pancreas	376	333	73·6	65·2	0·3	0·02	0·02
Liver	182	178	35·6	34·9	3·3	0·12	0·12
Salivary gland	127	294	24·9	57·6	0·3	0·01	0·02
Spleen	194	150	38·0	29·4	0·4	0·02	0·01
Brain	29	21	5·7	4·1	0·4	0·002	0·002
Lung	104	142	20·4	27·8	3·0	0·06	0·08
					Total	0·23	0·25
Total recovery of berberine (assuming average rats):						0·4%	0·4%

(b) Rat No. 3 injected intravenously with 5 mg (11·5 μc)
Rat No. 4 injected intravenously with 10 mg (23·0 μc)

Tissue	Specific activity, DPM H^3/mg tissue		Berb.(H^3).Cl. $2H_2O(H^3)$, μg/g tissue		Total dry tissue in average rat, g	Berb.(H^3).Cl. $2H_2O(H^1)$, mg/total, average tissue	
	Rat No. 3	Rat No. 4	Rat No. 3	Rat No. 4		Rat No. 3	Rat No. 4
Pancreas	1231	3010	241	589	0·3	0·07	0·18
Liver	316	1670	62	327	3·3	0·20	1·08
Salivary gland	1220	1140	239	223	0·3	0·07	0·07
Spleen	166	466	33	91	0·4	0·01	0·04
Brain	244	314	44	61	0·4	0·02	0·02
Lung[a]	6510	9420	1275	1844	3·0	3·8	5·5
					Total	4·2	6·9
Total recovery of berberine (assuming average rats):						84%	69%

[a] The extremely large amount of berberine in the lungs after the intravenous injections was frequently observed in other rats. Since berberine is very insoluble in any aqueous medium containing excess chloride and certain other negative ions, it will tend to precipitate out of solution when it reaches the saline in blood plasma with resultant deposit in the lungs.

only a very small quantity of berberine in the total pancreas, the maximum being around 0·18 mg. At the particular times chosen to sacrifice the animals after injection (2 to 5 min for intravenous, 4·5 h for subcutaneous injections) the intravenous method of administering berberine resulted in considerably higher concentrations in almost all organs tested, although the relative proportions of berberine in the organs of each rat remained roughly constant for both methods.

II. Observations Concerning Fluorescence of Berberine and Related Compounds in Animal Tissues

A. Quenching of Fluorescence

The bright fluorescence of the pancreas following injection of berberine into mice and rats initially suggested selective pancreatic localization of this and related alkaloids. As our results show, fluorescence is a very sensitive phenomenon and a poor criterion for the presence of appreciable quantities of a compound in animal tissue. The pancreata of all the rats that we have injected with berberine showed the characteristic bright yellow fluorescence, which was absent or present only dimly in other organs in the abdominal region. On analysis, however, it was found that berberine was present to some extent in all organs tested, and that the pancreas contained only a little more berberine per gram of tissue than the liver. We conclude, as did Blau and Bender,[15, 16] that this is probably due to quenching of fluorescence in these other organs. There are several possible reasons for this quenching phenomenon. For example, the yellow colour of fluorescing berberine could be absorbed by such organs as the liver, spleen, and heart, as indicated by their purple colour, whereas the yellow-white colour of pancreatic tissue would imply no such effect. Also, we have found that the fluorescence of berberine is markedly quenched by ferric ions and by haemoglobin. This could be an important factor in the liver which contains, in most species, two to ten times the iron concentration of the pancreas.[23]

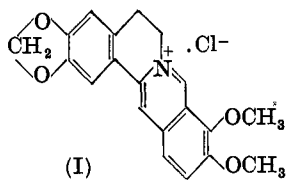
B. Fluorescence of Pancreatic Adenocarcinoma of the Hamster

Several primary tumours of hamster origin, among which are hepatoma, liposarcoma, and pancreatic adenocarcinoma, were

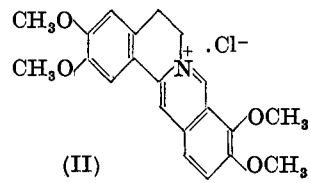
obtained from Dr. Joseph Fortner of the Memorial Center in New York City. These tumours were propagated in the hamster by means of subcutaneous transplantation. Animals bearing such subcutaneous tumours, and, in addition, animals with laboratory created sterile abscesses were injected with berberine chloride either subcutaneously or intravenously. The lesion was then exposed surgically and subjected to light from an ultraviolet source. Of the above mentioned lesions, none but the pancreatic adenocarcinoma exhibited the bright yellow fluorescence characteristic of berberine, although the pancreas of each hamster was observed to be fluorescent.

C. Observations on Pancreatic Fluorescence with Related Compounds

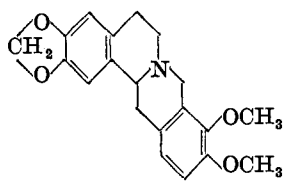
Nardi and Seipel[11] had found that palmatine (II), an alkaloid with structure and properties very similar to berberine (I), also caused fluorescence in the pancreas of rats and mice. On the



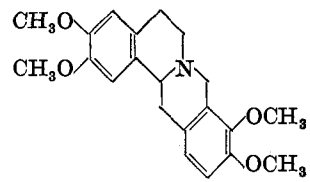
(I)



(II)



(III): Tetrahydroberberine

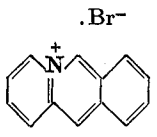


(IV): Tetrahydropalmatine

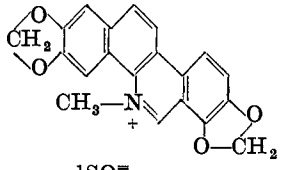
other hand, they observed no pancreatic fluorescence when the two alkaloids were used in the partially reduced, or tetrahydro, forms (III) and (IV). We have found that brilliant pancreatic fluorescence is produced with an iodinated berberine derivative, 9-demethyl-9-*p*-iodobenzylberberine chloride, (IX see part III), a compound which shows considerably different solubility in water when compared with berberine, although structurally the two are

closely related. Another berberine derivative, berberrubine (VII, see part III), was tested as the acid sulphate salt and also found to produce fluorescence in the pancreas of a 300 g rat when 9 mg was injected intravenously in 1 per cent aqueous solution.

It was interesting to test other available fluorescent compounds which contain a quaternary nitrogen group and have some structural similarity to berberine. The first compound investigated was acridizinium bromide (V),* which shows a blue fluorescence under ultraviolet light. Three rats, weighing 320 g, 250 g, and 300 g, were injected intravenously with 2·5, 1·5, and 1·3 mg, respectively, of this compound in a 1 per cent aqueous solution. All of the animals died 5 to 13 min after injection and were autopsied immediately. No pancreatic fluorescence was observed. By



(V)



(VI)

way of comparison it should be noted that berberine produces marked fluorescence in the pancreas within one minute after intravenous injection.

We also tested the alkaloid, sanguinarine sulphate (VI)†, which has a structure rather more similar to that of berberine. The sulphate salt exhibits an orange to red fluorescence while the hydroxide form has a bluish-violet fluorescence. Three rats, weighing 270 to 290 g, were injected intravenously with 10 mg of sanguinarine sulphate as a 1 per cent aqueous solution. Respiratory stimulation was observed during injection, but respiration quickly returned to normal. Some fluorescence was observed in the kidneys, livers, duodena, and small and large intestines, but none was seen in the pancreata when the animals were sacrificed about thirty minutes after injection.

* A sample of acridizinium bromide was generously provided by Prof. C. K. Bradsher of Duke University.

† Samples of sanguinarine sulphate and other alkaloids were obtained through the courtesy of S. B. Penick and Co., N.Y.

Of the small number of compounds tested to date, therefore, only those which contain the berberine ring system produce fluorescence in pancreatic tissue. Variations in the side chains appear to have little effect on this property.

III. Iodinated Derivatives of Berberine

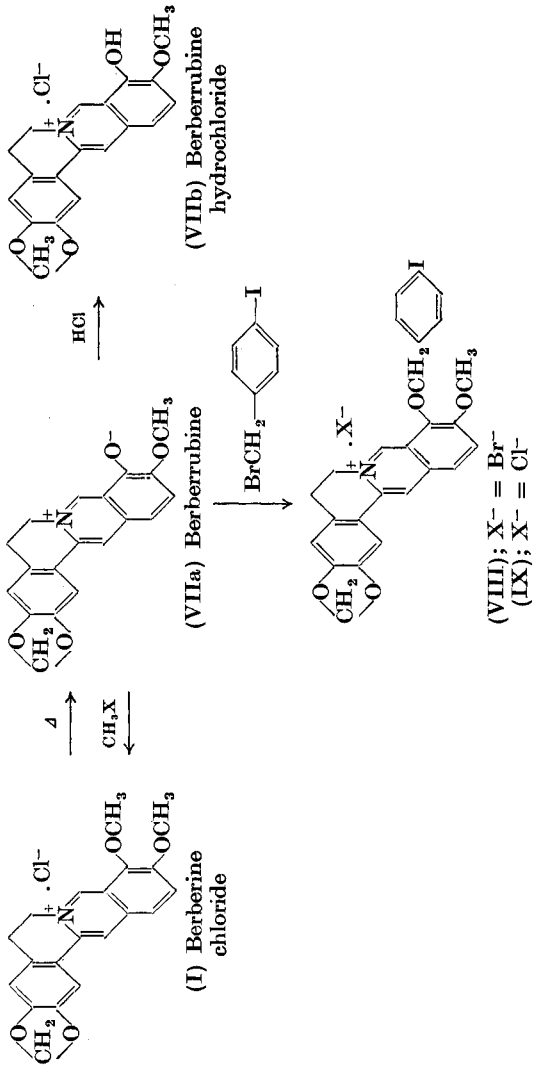
Although berberine (I) itself cannot be iodinated directly, it can be converted readily into berberrubine (VII).[24, 25] This compound, which in base is a phenol betaine (VIIa) and in acid, a phenol (VIIb), has been shown to give both a chloro- and a bromo-derivative on treatment with sodium hypochlorite or hypobromite.[25] We found, however, that berberrubine was recovered unchanged after treatment with sodium hypoiodite. No organically bound iodine was detected in the various products after berberrubine had been treated with iodine in the presence of ammonia, amines, or acetic acid–sodium acetate, or with iodine monochloride. A frequent cause of interference in these reactions was the formation of extremely insoluble iodide salts.

Heating berberrubine (VIIa) with a methyl halide reconverts it to berberine.[24] In an analogous reaction, we treated berberrubine with *p*-iodobenzyl bromide to obtain a berberine derivative with an iodinated side chain. The expected product, 9-demethyl-9-*p*-iodobenzylberberine bromide (VIII), was obtained in high yield.

Since the bromide salt (VIII) is very insoluble in water, other salts including the phosphate, sulphate, mucate, and chloride were investigated. Of these the chloride salt (IX) was chosen for biological testing, although its solubility is only 0·07 g/100 ml of water at 37°. As pure solids, both compounds (VIII and IX) are decidedly radiopaque compared to an equimolar quantity of pure berberine chloride. However, the chloride salt (IX) is not appreciably radiopaque when dissolved in propylene glycol up to a metastable concentration of 20 per cent by weight.

Testing was carried out by injecting a 1 per cent solution of the chloride salt (IX), usually in propylene glycol, intravenously in rats. The injection of 1 mg of this compound usually resulted in the death of a 300 g rat, although as much as 5 mg was administered in several cases before expiration. The pancreata

of these rats fluoresced a more brilliant yellow than any others we had seen. However, roentgenograms of the individual pancreas or of the whole rat showed no more radiopacity than the controls.



*Preparation of 9-demethyl-9-*p*-iodobenzylberberine bromide (VIII) and chloride (IX)*

Berberrubine, prepared by the method of Frerichs and Stoepel,[25] was dried for five hours at 100°/1 mm, then 2·00 g of it was finely powdered and mixed in a mortar with *p*-iodobenzyl bromide (3·16 g, 2:1 molar excess).[26] The mixture was placed in a 125-ml flask under a stream of dry nitrogen and heated in a boiling water bath with occasional shaking and stirring for about 30 min. The solid, which had turned yellow, was cooled and triturated several times with boiling ether, and finally washed on a filter with sufficient ether to remove the last traces of *p*-iodobenzyl bromide. The yield was 3·56 g (99·4 per cent) of crude product. Recrystallization of 1·46 g from absolute ethanol (375 ml) after treatment with decolorizing charcoal gave the pure product (VIII) (1·04 g) as yellow needles. The compound darkened in vacuum above 150° and melted above 215° (d.) depending on the rate of heating. An analytical sample was obtained after two more recrystallizations from ethanol.

Anal. Calcd. for $C_{26}H_{21}BrINO_4$: C, 50·51; H, 3·42. Found: C, 50·64; H, 3·42.

The chloride salt (IX) was prepared by dissolving the crude bromide (VIII) (1·00 g) in boiling absolute ethanol (226 ml), filtering with charcoal, and treating the warm solution with 6 N hydrochloric acid (90 ml) and concentrated hydrochloric acid (15 ml). Tiny balls of fine yellow needles formed slowly on cooling in a yield of 0·64 g. An analytical sample was obtained after recrystallization from ethanol (50 ml) with 6 N hydrochloric acid (24 ml) added during cooling. This material, which appears to be a dihydrate, decomposed on drying above 100°. It darkened in vacuum above 150° and melted at about 190° (d.) depending on the rate of heating.

Anal. Calcd. for $C_{26}H_{21}ClINO_4.2H_2O$: C, 51·21; H, 4·13. Found: C, 50·99; H, 4·15.

Conclusions

The plant alkaloid, berberine, is located, after either subcutaneous or intravenous injection, in small concentrations in many different organs of the rat. No evidence has been found to indi-

cate selective localization of berberine in the pancreas, although this organ alone shows the bright yellow fluorescence of berberine after injections. Therefore, we have been unable to confirm the results of Nardi and Seipel,[11] who reported high percentage recoveries of berberine from rat pancreata. This is in accord with the findings of Blau and Bender.[16]

Gravimetric methods of analysis sensitive to 0·5 mg of berberine, including the procedure of Nardi and Seipel, were found to be inadequate for detecting berberine in pancreatic tissue after injection. It was necessary to develop a far more sensitive spectrophotometric procedure to identify the small quantities of berberine present in liver, spleen, salivary gland, kidney, and brain, as well as pancreas of an injected animal. Rats sacrificed at various times following either intravenous or subcutaneous injections showed highest concentrations of berberine at the shortest time intervals studied with no indication of any build-up or retention of berberine in the pancreas, liver, or other organs.

These conclusions have been confirmed by radio-assay of the tissues of four rats injected under various conditions with tritium-labelled berberine. The distribution of berberine in the tissues of these animals was qualitatively similar to that found in rats studied more extensively by the spectrophotometric procedure. Largest amounts of berberine are found in the total liver, which contains two to six times as much as the total pancreas. A maximum of 0·18 mg of berberine was identified in the pancreas from an injected dose of at least 10 mg.

Although berberine fluorescence appears concentrated mainly in pancreatic tissue of rats, mice,[11,12] and hamsters, this observation gives little indication of the quantity of berberine actually present in any region. Minute amounts of berberine are often clearly visible, whereas the fluorescence of far greater concentrations can be masked easily by other colours or quenched by agents such as iron. The property of fluorescence following berberine injection is observed also in pancreatic adenocarcinoma of the hamster even when the tumour is transplanted subcutaneously to other hamsters. Similar fluorescence did not occur in other types of tumours when implanted subcutaneously.

Of compounds tested, only those containing the berberine ring system have been found to produce pancreatic fluorescence.

Other aromatic quaternary nitrogen compounds like acridizinium bromide and sanguinarine sulphate, as well as the tetrahydro forms of berberine and palmatine,[11] do not exhibit this phenomenon.

A berberine derivative containing an iodinated side chain, 9-demethyl-9-*p*-iodobenzylberberine bromide, has been prepared by treating berberrubine with *p*-iodobenzyl bromide. The chloride salt of this compound, although much more toxic and less soluble in water than berberine, produces brilliant pancreatic fluorescence in rats. However, there was no radiopacification of the pancreas following injection of this material.

Summary. A report[11] of selective pancreatic localization of berberine suggested the possibility of iodination of this and similar compounds for use in pancreatic roentgenography. A study has been made of the tissue distribution, fluorescent properties, and iodination of berberine. Gravimetric, spectrophotometric, and radio-isotope assay procedures for determining berberine in animal tissues have been developed. Small amounts of berberine were identified in rat pancreas, liver, salivary gland, spleen, kidney, brain, and lung, following intravenous or subcutaneous injection. However, no evidence to confirm selective localization of berberine in the pancreas has been found, although bright yellow fluorescence from berberine is observed mainly in pancreatic tissue.

A subcutaneously transplanted pancreatic adenocarcinoma of the hamster also showed berberine fluorescence, a phenomenon not observed in subcutaneous hepatomas, liposarcomas, and sterile abscesses.

Other alkaloids containing the berberine ring system produce pancreatic fluorescence, whereas tetrahydroberberine,[11] tetrahydropalmatine,[11] acridizinium bromide, and sanguinarine sulphate do not.

An iodinated berberine derivative, 9-demethyl-9-*p*-iodobenzylberberine chloride, has been prepared and found to produce pancreatic fluorescence but no radiopacification in rats.

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