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CYKL PENTOZOWY U MACZUGOWCÓW BŁONICY

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Przemiana węglowodanowa maczugowców błonicy jest mało zbadana. Holdsworth [9], Gubariew [7], Orłowa [18], Kwapiński [12] zajmowali się raczej budową występujących cukrów, a nie ich przemianą. Fujita i Kodama w roku 1934 [6] stwierdzili powstawanie kwasu mlekowego z glukozy. Praca ta nie daje jednak obrazu przemiany węglowodanowej u maczugowców błonicy. Łatwość metabolizowania rybozy, jaką stwierdzono u *Corynebacterium diphtheriae* [2], oraz rozpowszechnienie występowania cyklu pentozowego w świecie bakterii [19, 22, 8] nasunęły myśl zbadania występowania tej drogi metabolicznej u maczugowców błonicy.

MATERIAŁ I METODY

Do badań użyto *Corynebacterium diphtheriae* szczep PW8 otrzymany z PZH, przechowywany na stałym podłożu Loefflera. Bakterie hodowano przez 4 dni na pożywce Mueller-Miller z dodatkiem 0,0024 mg % tiaminy, w temperaturze 34° [16]. Do sporządzania pożywki używano handlowego kwaśnego hydrolizatu kazeiny.

W pracy stosowano dwa rodzaje preparatów enzymatycznych. Początkowo z nie rozbitych komórek bakterii przygotowywano proszek acetonowy. W późniejszych doświadczeniach bakterie rozcierano z proszkiem szklanym, a otrzymany wyciąg poddawano różnicowemu wirowaniu. Z proszków acetonowych otrzymywano preparaty enzymatyczne przez ekstrahowanie buforem i wytrącanie $(NH_4)_2SO_4$ w niskiej temperaturze [3]. Zarówno masę bakteryjną, jak i proszek acetonowy można przechowywać w temperaturze —18° przez szereg miesięcy bez straty aktywności.

Do 20 g bakterii dodawano 40 g proszku szklanego i z 3—4 ml 0,15 M-KCl przygotowywano gęstą papkę, którą rozcierano w homogenizatorze Pottera-Elvehjema przy intensywnym chłodzeniu z zewnątrz.

Ilość szklanego proszku posiada istotne znaczenie dla rozbicia błony komórkowej. Stopień rozbicia bakterii badano w mikroskopie fazowym. Po paru doświadczeniach stwierdzono, że intensywne 20-minutowe rozcieranie w homogenizatorze zapewnia należyte rozbicie bakterii. Do roztartych bakterii dodawano równą objętość 0,15 M-KCl i przeprowadzano różnicowe wirowanie w niskiej temperaturze. Proszek szklany, resztki komórkowe i nie rozbite bakterie osadzano przy przyśpieszeniu 800 *g* przez 20 min. Frakcję odpowiadającą mitochondriom osadzano stosując przyśpieszenie 8500 *g* przez 10 min. Trzecie i ostatnie wirowanie przeprowadzono stosując przyśpieszenie 18 500 *g* przez 30 min. Otrzymane osady przy drugim i trzecim wirowaniu przepłukiwano dwukrotnie 0,15 M-KCl. Przepłukane osady zawieszano w 0,15 M-KCl. Żółtawy i lekko opalizujący płyn, który pozostawał po wirowaniach, nazwano frakcją S.

Ze względu na to, że cykl pentozowy w tkankach zwierzęcych jest dokładnie poznany, dla łatwiejszej interpretacji wyników przeprowadzono doświadczenia, w których użyto preparat otrzymany z wątroby królika przez frakcjonowane wirowanie [17].

Rybozo-5-fosforan (R5P)[1] otrzymano przez hydrolizę kwasu adenilowego z mięśni [10]. Czystość otrzymanego preparatu wynosiła 77,5% na podstawie oznaczenia fosforu i pentozy [13].

Glukozo-6-fosforan otrzymano za pomocą fosfoglukomutazy z glukozo-1-fosforanu [21]. Glukozo-1-fosforan preparowano ze skrobi przy użyciu świeżego soku ziemniaczanego [15]. 6PG otrzymywano przez utlenianie G6P bromem [20]. W niektórych doświadczeniach używano preparatu handlowego R5P i G6P firmy Nutritional Biochemical Corp. DPN i TPN były preparatami handlowymi tej samej firmy.

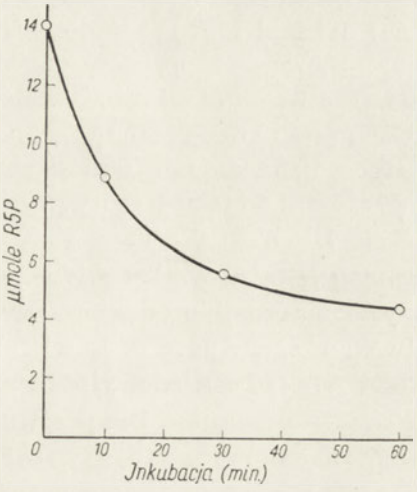
Pentozy oznaczano metodą Mejbaum [13], czas ogrzewania we wrzącej łaźni wodnej wynosił 40 minut. Ketozy oznaczano metodą cysteino-karbazolową Dischego [1]. Jako standardu używano *o*-nitrofenylohydrazonu rybulozy otrzymanego z laboratorium National Institute of Health, Bethesda, dzięki uprzejmości dr G. Ashwella. Białka oznaczano metodą Lowry i wsp. [14].

WYNIKI

W pierwszym etapie pracy zbadano szybkość zużywania R5P przez preparat enzymatyczny otrzymany z proszku acetonowego. Wyniki przedstawiono na rys. 1.

[1] W pracy użyto następujących skrótów: R5P — rybozo-5-fosforan, G6P — glukozo-6-fosforan, 6PG — kwas 6-fosfoglukonowy, ATP — adenozynotrójfosforan, TPN — trójfosfopirydynonukleotyd, DPN — dwufosfopirydynonukleotyd, Ru5P — rybulozo-5-fosforan, TCA — kwas trójchlorooctowy.

Następnie przystąpiono do zbadania lokalizacji enzymów cyklu pentozowego we frakcjach komórkowych otrzymanych przez różnicowe wirowanie. Lokalizację ustalono na podstawie pomiaru zużycia R5P i wytwa-



Rys. 1. Zużycie R5P przez preparat enzymatyczny z proszku acetonowego *C. diphtheriae*. 14,2 µmoli R5P, 6 µmoli $MgCl_2$, 0,2 ml buforu weronalowego o pH 7,6 i 0,3 ml preparatu enzymatycznego zawierającego 4,1 mg białka. Temp. 37°. Na początku doświadczenia oraz w określonych odstępach czasu pobierano po 0,2 ml płynu i odbiałczano 9,8 ml 0,3 M-TCA. Po przesączeniu oznaczano pentozy metodą Mejbaum

rzania ketoz pod wpływem różnych frakcji. Jak widać z wyników podanych w tablicy 1, największą aktywność ma frakcja S.

Tablica 1

Lokalizacja enzymów nieoksydatywnych cyklu pentozowego we frakcjach komórkowych

Inkubowano w 37°: 10 µmoli R5P, 6 µmoli $MgCl_2$, 0,2 ml buforu glicyl-glicynowego o pH 7,6 i 0,2 ml danej frakcji. Po 30 minutach pobierano próbki po 0,2 ml do oznaczenia pentoz i ketoz. Do oznaczenia pentoz próbki odbiałczano 0,3 M-kwasem trójchlorooctowym, a ketoz — 0,9 M-$HClO_4$.

Frakcja osadzana przy	Białko (mg/ml frakcji)	Δ pentoz (µmole/mg białka)	Δ ketoz (µmole/mg białka)
8 500 *g*	7,7	0,58	0,13
18 500 *g*	4,6	0,54	0,0
płyn znad osadu (frakcja S)	10,9	2,11	0,46

W tablicy 2 przedstawiono przebieg zużycia R5P i wytwarzania ketoz pod wpływem frakcji S.

Podczas badania produktów przemiany R5P stwierdzono, że z R5P poprzez produkty pośrednie powstaje sedoheptuloza, którą oznaczano metodą Dischego [4] na podstawie spektrofotometrycznego pomiaru absorpcji produktów reakcji sedoheptulozy z cysteiną i H_2SO_4 przy 520 mμ. Skład mieszaniny inkubowanej był taki sam jak w doświadczeniach, w których badano zużywanie R5P. Stosowano preparat enzymatyczny otrzymany z proszku acetonowego *C. diphtheriae*. Próbki w ilości 0,2 ml pobierano w odpowiednich odstępach czasu i dopełniano wodą do 2 ml. Celem zatrzymania reakcji enzymatycznej mieszaninę umieszczano we wrzącej łaźni wodnej na 3 min. Po ostudzeniu do temperatury pokojowej pobierano 1 ml i przeprowadzano reakcję Dischego. Pomiary absorpcji wykonywano na spektrofotometrze Beckman DU. Otrzymane widma absorpcyjne przedstawiono na rys. 2. Krzywe absorpcji produktów powstających w reakcji Dischego wykazują dwa maksima: przy długości fali 410 mμ i 520 mμ. Maksimum przy długości fali 410 mμ odpowiada produktom, które powstają z heksoz, przy 520 mμ — z heptuloz. Dla porównania przeprowadzono doświadczenie z zastosowaniem preparatu z wątroby królika (Rys. 3), w której przemiana pentozowa jest dokładnie poznana.

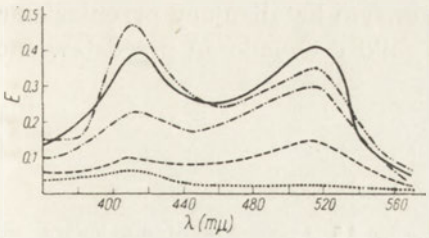
Tablica 2

Szybkość zużywania R5P i wytwarzania ketoz przez frakcję komórkową o największej aktywności

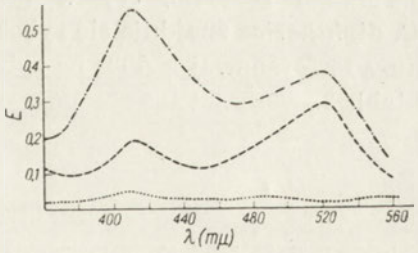
70 μmoli R5P inkubowano w temp. 37° z 18 μmolami $MgCl_2$ w 1 ml buforu glicyl-glicynowego o pH 7 z 0,5 ml frakcji S (zawartość białka 7,5 mg). Odbiałczenie jak podano w tablicy 1.

Inkubacja (min.)	Pentozy (μmole)	Ketozy (μmole)
0	68,8	8,2
1	63,2	10,8
2	63,0	12,1
3	61,4	13,3
4	56,3	13,6
5	54,1	15,2
10	45,1	17,7
15	33,8	19,3
30	32,9	19,1

W drugiej części pracy badano występowanie we frakcji S enzymów dehydrogenazy G6P i 6PG, odpowiedzialnych za utlenianie G6P do 6PG i z kolei za utlenianie kwasu 6-fosfoglukonowego do pentozy z wydzieleniem CO_2. Początkowe doświadczenia miały na celu stwierdzenie, czy dehydrogenazy te współdziałają z DPN, jak bywa u niektórych bakterii,

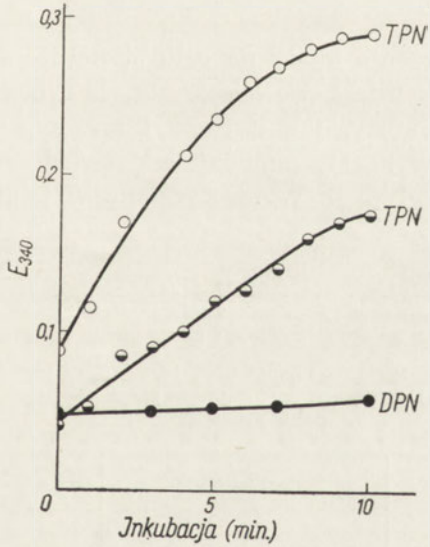


Rys. 2. Widma absorpcyjne produktów reakcji Dischego. Preparat enzymatyczny z *C. diphtheriae*. Skład inkubatu jak podano w rys. 1. Czas inkubacji: (••••) 0 min.; (- - -) 10 min.; (-•-) 30 min.; (——) 60 min.; (-••-) 90 min.



Rys. 3. Widma absorpcyjne produktów reakcji Dischego. Preparat enzymatyczny z wątroby królika. Skład inkubatu jak podano w rys. 1. Czas inkubacji: (••••) 0 min.; (- - -) 10 min.; (-•-) 30 min.

czy też z TPN, który jest powszechnie znanym koenzymem tych dehydrogenaz. Wyniki doświadczeń przedstawiono na rys. 4, i jak widać, redukcja DPN jest znikoma. Doświadczenia, w których zamiast DPN użyto TPN, wykazały szybki i znaczny wzrost absorpcji niezależnie od tego, czy jako substrat zastosowano G6P czy 6PG.



Rys. 4. Redukcja DPN i TPN pod wpływem dehydrogenaz G6P i 6PG *C. diphtheriae*. Inkubowano: 0,15 μmola nukleotydu, 10 μmoli G6P (lub 18 μmola 6PG), 0,2 μmola $MgCl_2$ z 0,1 ml frakcji komórkowej nie osadzającej się przy 18 500 *g* (zawartość białka 1,44 mg) w buforze glicyl-glicynowym o pH 7,6. Końcowa objętość 3 ml., temp. 22°. Próba ślepa nie zawierała substratu. (○), glukozo-6-fosforan, (◒), kwas fosfoglukonowy, (●), glukozo-6-fosforan

Ponadto zbadano, czy w otrzymywanych frakcjach komórkowych z *C. diphtheriae* znajduje się rybokinaza, enzym katalizujący przeniesienie grupy fosforanowej z ATP na rybozę. Wyniki doświadczeń przedstawiono w tabl. 3.

Tablica 3

Rybokinaza we frakcjach komórkowych

Inkubowano w 25°: 0,2 μmole rybozy, 10 μmoli $MgCl_2$, 4.5 μmola ATP, 1 ml buforu tris o pH 7,5 i 0,2 ml preparatu enzymatycznego. Próbki po 0,2 ml pobierano na początku inkubacji i po upływie 30 min. Próbki odpipetowano do 1 ml alkoholu etylowego 96% zawierającego 0,02 ml 2 M-$BaCl_2$. Powstały ester R5P ulega wytrąceniu jako sól barowa. Pobierano 0,4 ml płynu znad osadu do oznaczania zawartości pentozy.

Frakcja osadzana przy	Zawartość białka w mieszaninie inkubowanej (mg/ml)	Pentozy (μmole)		Utworzony R5P (μmole/mg białka)
		0′	30′	
8 500 *g*	1,5	2,3	2,1	0,10
18 500 *g*	0,9	1,9	1,8	0,16
Płyn znad osadu (frakcja S)	2,2	2,1	1,1	0,42

Następne doświadczenia miały na celu stwierdzenie, czy pentokinaza w maczugowcach błonicy jest swoiście działającą rybokinazą, czy też może estryfikować inne pentozy, jak ksylozę i arabinozę. Do badań użyto frakcji S, która jak wykazały poprzednie doświadczenia wykazuje największą zdolność estryfikacji. Wyniki podano w tablicy 4.

Tablica 4

Swoistość pentokinazy zawartej we frakcji komórkowej o największej aktywności

0,2 μmola pentozy, 10 μmoli $MgCl_2$, 4,5 μmoli ATP, 1 ml buforu tris o pH 7,5 i 0,2 ml frakcji S (zawartość białka 2,19 mg) inkubowano 30 min. w temp. 25°. Odbiałczano i oznaczano jak podano w tablicy 3.

Substrat	Pentozy (μmole)		Zestryfikowana pentoza (μmole)
	0′	30′	
ryboza	2,1	1,1	1,0
arabinoza	2,2	2,2	0,0
ksyloza	2,2	2,2	0,0

DYSKUSJA I WNIOSKI

Wyniki otrzymane w tej pracy przemawiają za udziałem cyklu pentozowego w przemianie węglowodanowej *C. diphtheriae*. Badania wykazały jakościową zgodność przemiany R5P w preparatach enzymatycznych z badanych bakterii i z wątroby królika.

Z pracy Newburgh'a i Cheldelin'a [17] wiadomo, że enzymy nieoksydatywne nie są związane ze strukturą komórkową. Podobnie zlokalizowane są również enzymy cyklu pentozowego u maczugowców błonicy. Wyniki przedstawione w tablicy 1 wykazują, że enzymy te są rozpuszczone w soku komórkowym, a nie związane ze strukturą komórkową. Znikanie dodanego R5P do preparatów z *C. diphtheriae* i powstawanie ketoz pozwala wnioskować o obecności fosfopentoizomerazy przekształcającej R5P w Ru5P. Otrzymane maksimum przy 520 mμ w reakcji Dischego, odpowiadające sedoheptulozie, sugeruje obecność w preparatach epimerazy i transketolazy. Spadek absorpcji przy 520 mμ w czasie inkubacji przy jednoczesnym wzroście maksimum przy 410 mμ nasuwa przypuszczenie obecności transaldolazy przerzucającej trójwęglowy fragment z sedoheptulozy na fosfotriozę z wytworzeniem fosfoheksozy.

Redukcja TPN w czasie inkubacji G6P lub 6PG z frakcją S świadczy o obecności dehydrogenaz. Brak redukcji DPN przemawia za faktem, iż specyficznym koenzymem dla tych dehydrogenaz jest TPN, chociaż wiadomo, że niektóre dehydrogenazy G6P i 6PG pochodzenia bakteryjnego reagują z DPN [3].

Badania obecności enzymu pentokinazy dały wynik pozytywny. Preparat enzymatyczny, inkubowany z rybozą i ATP, posiadał zdolność fosforylowania jej. Dalsze doświadczenia wykazały, że mamy do czynienia ze swoiście działającą rybokinazą, ponieważ ani ksyloza, ani arabinoza w czasie inkubacji nie ulegały fosforylacji. Rybokinaza, podobnie jak inne enzymy cyklu pentozowego, nie jest związana ze strukturą komórkową, lecz rozpuszczalna w soku komórkowym.

STRESZCZENIE

Preparaty enzymatyczne z *Corynebacterium diphtheriae* zużywały w czasie inkubacji R5P z wytwarzaniem ketoz. Identyfikację dalszych produktów przemiany R5P przeprowadzono spektrofotometrycznie przy pomocy reakcji Dischego. Stwierdzono powstawanie sedoheptulozy i przechodzenie jej w heksozy. Pozwala to wnioskować o istnieniu w maczugowcach następujących enzymów: izomerazy fosfopentoz, epimerazy, transketolazy i transaldolazy.

Zbadano lokalizację enzymów we frakcjach komórkowych otrzymanych przez frakcjonowane wirowanie rozbitych komórek bakteryjnych. Błonę komórkową zniszczono przez rozcieranie bakterii z proszkiem szklanym. Wykazano obecność swoiście działającej rybokinazy. Stwierdzono obecność dehydrogenaz G6P i 6PG.

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THE PENTOSE CYCLE IN *CORYNEBACTERIUM DIPHTHERIAE*

Summary

The enzymatic preparations of *Corynebacterium diphtheriae* consumed R5P and produced some keto-sugars on incubation. The identification of the further R5P metabolites was performed spectrophotometrically by means of the Dische reaction. The formation of sedoheptulose was stated, as well as its transition into hexose. This observation allowed to conclude that the following enzymes existed in *Corynebacteria:* phosphopentose isomerase, epimerase, transketolase and transaldolase.

The localization of the enzymes was investigated in the cell fractions obtained on differential centrifugation of the disrupted cells. The cell membrane was destroyed by grinding the bacteria with glass powder. The presence of the specific ribokinase was detected, as well as that of G6P and 6PG dehydrogenases.

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BIOSYNTEZA I PRZEMIANY ALKALOIDÓW W ŁUBINIE WĄSKOLISTNYM

II. BIOSYNTEZA ALKALOIDÓW W IZOLOWANYCH ZARODKACH I LIŚCIENIACH

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Kierownik: prof. dr Ignacy Reifer

W naszej poprzedniej pracy [6] nad zmianami składu alkaloidów we wczesnych stadiach rozwoju łubinu wąskolistnego wykazano, że w pierwszych dniach kiełkowania nasion ogólna ich zawartość niemal nie zmienia się, natomiast poszczególne alkaloidy w liścieniach ulegają wzajemnym przekształceniom. Intensywną biosyntezę alkaloidów zaobserwowano dopiero po upływie 4 dnia rozwoju rośliny.

Niniejsza praca przedstawia wyniki doświadczeń, w których badano zdolność syntezy alkaloidów w oddzielonych zarodkach i liścieniach.

MATERIAŁY I METODY

Materiałem doświadczalnym były izolowane zarodki i liścienie oraz młode rośliny łubinu wąskolistnego, które hodowano w jałowych kulturach z nasion odmiany „Wielkopolski gorzki".

Kultury jałowe zakładano w sposób podany w I części pracy [6], przy czym kiełkowanie nasion odbywało się w temperaturze około 25°. W celu zachowania jednakowych warunków oświetlenia (doświadczenia były wykonane w różnych porach roku) hodowano kultury w temperaturze 27° w szafie Neubauera zaciemnionej od strony słonecznej, stosując na dobę 12-godzinne naświetlanie za pomocą dwu żarówek 100-watowych.

Natychmiast po pobraniu prób analizowano świeży materiał roślinny, z którego ekstrahowano alkaloidy metodą Reifera i Niziołka [3]. Poszczególne alkaloidy po rozdziale chromatograficznym oznaczano metodą ko-

lorymetryczną [5] (Tabl. 3 i 4), a w następnych doświadczeniach — metodą nefelometryczną [4]. Podobnie jak w poprzedniej pracy [6], zawartość alkaloidów wyrażano w przeliczeniu na jedną roślinę lub izolowany organ.

W przypadku zarodków izolowanych, zarodków z obok leżącymi liścieniami i nie naruszonych roślin podawano w tablicach średnie wartości z dwóch oznaczeń równoległych, przy czym wyniki tych oznaczeń różniły się nie więcej niż o 10%. W przypadku doświadczeń z izolowanymi liścieniami zastosowano metodę tzw. „połówek", co pozwoliło na uzyskanie bardzo zgodnych wyników. W metodzie tej każda próba „pełna" posiadała odpowiadającą jej próbę kontrolną. Na jedną próbę zarówno pełną, jak i kontrolną przypadało po 10 liścieni. Równoległe próby „połówkowe" uzyskiwano, wykorzystując jeden z dwu liścieni każdego nasienia jako kontrolę, a drugi jako kulturę doświadczalną.

WYNIKI

W celu sprawdzenia wcześniejszej obserwacji Reifera i Kleczkowskiej [2], że zarodki izolowane po jednym dniu od momentu namoczenia nasion nie są zdolne do samodzielnego syntezowania alkoloidów, w pracy tej powtórzono doświadczenie z takimi zarodkami. Dodatkowo porównano zmiany zawartości alkaloidów w zarodkach ze zmianami w liścieniach izolowanych w tym samym czasie i hodowanych oddzielnie. Okazało się, że istotnie suma alkaloidów nie wzrasta w izolowanych po 1 dniu zarodkach (Tabl. 1), lecz utrzymuje się do 7 dnia rozwoju na tym samym po-

Tablica 1

Zmiany zawartości alkaloidów w zarodku hodowanym po oddzieleniu liścieni w 1 dniu (od momentu namoczenia nasion)

Nr doświadczenia	Dzień rozwoju po oddzieleniu liścieni	Zawartość alkaloidów (w % sumy alkaloidów w kontroli)			
		X_1*	lupanina	hydroksylupanina	suma alkaloidów
I	0	0,0	48,9	51,1	100,0**
	7	0,0	77,8	28,8	106,6
	14	0,0	67,6	21,0	88,6
II	0	13,2	43,4	43,4	100,0***
	7	0,0	77,8	25,1	102,9

* Nie zidentyfikowana substancja reagująca z odczynnikiem Dragendorffa.
** Suma alkaloidów w zarodku kontrolnym wynosiła 31,0 µg.
*** Suma alkaloidów w zarodku kontrolnym wynosiła 33,3 µg.

ziomie, a po 14 dniach nawet wyraźnie obniża się. W izolowanych po 1 dniu liścieniach (Tabl. 2) stwierdzono, że suma alkaloidów nieco wzrasta w ciągu 6 dni hodowli, a więc liścienie takie posiadają zdolność samodzielnego syntezowania alkaloidów.

Tablica 2

Zmiany zawartości alkaloidów w liścieniach hodowanych po oddzieleniu zarodka w 1 dniu

Nr doświadczenia	Dzień rozwoju po oddzieleniu zarodka	Zawartość alkaloidów (w % sumy alkaloidów w kontroli)					
		X_1	lupanina	hydroksylupanina	oksylupanina	angustyfolina	suma alkaloidów
I	0	0,0	47,7	50,5	0,0	1,8	100,0*
	6	ślady	52,9	51,1	ślady	12,8	116,8
II	0	0,0	48,6	44,8	0,0	6,6	100,0**
	6	0,0	52,4	44,0	11,6	16,2	124,2

* Suma alkaloidów w liścieniu kontrolnym wynosiła 1089 µg.
** Suma alkaloidów w liścieniu kontrolnym wynosiła 1050 µg.

Omówione wyniki zgodnie ze wspomnianą pracą [2] wskazują, że zdolność młodej rośliny do syntezowania alkaloidów, przynajmniej w pierwszych dniach rozwoju jest zależna od obecności liścieni. Wydawało się zatem, że celowe byłoby doświadczalne wykazanie istnienia takiego

Tablica 3

Zmiany w zawartości alkaloidów w zarodkach hodowanych po oddzieleniu liścieni w 7 lub 2 dniu

Nr doświadczenia	Dzień oddzielenia liścieni	Dzień rozwoju po oddzieleniu liścieni	Zawartość alkaloidów (w % sumy alkaloidów w kontroli)				
			lupanina	hydroksylupanina	oksylupanina	angustyfolina	suma alkaloidów
I	7	0	51,2	36,5	12,3	0,0	100,0*
		6	45,3	58,4	21,8	0,0	125,5
II	2	0	51,7	36,0	12,3	0,0	100,0**
		6	46,0	35,7	0,0	0,0	81,7
III	2	0	46,7	47,4	0,0	5,9	100,0***
		3	45,5	33,4	ślady	11,5	90,4
		6	41,1	34,6	7,6	8,0	91,3
		10	50,1	35,0	9,5	7,1	101,7
		12	49,1	37,9	7,0	6,1	100,1
		15	29,0	26,5	6,8	6,4	68,7

* Suma alkaloidów w zarodku kontrolnym wynosiła 876,0 µg.
** Suma alkaloidów w zarodku kontrolnym wynosiła 156,2 µg.
*** Suma alkaloidów w zarodku kontrolnym wynosiła 58,9 µg.

okresu rozwoju zarodka z nie oddzielonymi liścieniami, po upływie którego izolowany zarodek mógłby już samodzielnie syntezować alkaloidy.

W tym celu wykonano serię doświadczeń (Tabl. 3), w których przyjęto dwa znacznie różniące się okresy do momentu oddzielenia liścieni, tj. 2 i 7 dni. W zarodkach izolowanych w 7 dniu (Tabl. 3, doświad. I) obserwuje się po dalszych 6 dniach hodowli zdolność syntezowania alkaloidów, natomiast zarodki izolowane w 2 dniu (doświad. II) wykazują w tym samym czasie spadek absolutnej zawartości alkaloidów. W doświadczeniu III analizowano 2-dniowe zarodki w ciągu dłuższego okresu hodowli i w krótkich odstępach czasu. Okazało się, że istotnie nie wykazują one zdolności syntezy alkaloidów. Wynika stąd, że poszukiwany okres, w ciągu którego niezbędna jest obecność liścieni przy zarodku, znajduje się pomiędzy 2 i 7 dniem rozwoju rośliny (w przyjętych warunkach).

Tablica 4

Zmiany w zawartości alkaloidów w zarodkach hodowanych po oddzieleniu liścieni w 4 dniu

Nr doświadczenia	Dzień rozwoju po oddzieleniu liścieni	Zawartość alkaloidów (w % sumy alkaloidów w kontroli)					
		X_1	lupanina	hydroksy-lupanina	oksy-lupanina	angusty-folina	suma alkaloidów
I	0	7,3	43,3	37,0	0,0	12,4	100,0*
	7	4,3	92,2	52,2	23,9	26,4	199,0
II	0	0,0	71,3	28,7	0,0	0,0	100,0**
	7	0,0	94,2	25,6	27,0	28,5	175,3

* Suma alkaloidów w zarodku kontrolnym wynosiła 292,0 µg.
** Suma alkaloidów w zarodku kontrolnym wynosiła 534,3 µg

Ponieważ z I części pracy [6] wynikało jednak, że młoda roślina — rozwijająca się z liścieniami — przechodzi okres początkowej „organizacji", po którym dopiero obserwuje się wzrost zawartości alkaloidów, postanowiono zatem przebadać zarodki i liścienie izolowane bezpośrednio po tym właśnie okresie, tj. po 4 dniach wstępnej hodowli. W kolejnym doświadczeniu wykazano (Tabl. 4), że zarodki izolowane z 4-dniowych roślin są już zdolne syntezować alkaloidy, których suma w porównaniu do kontroli wzrasta po dalszych 7 dniach hodowli o 75—100%. Również liścienie izolowane w 4 dniu (Tabl. 5) przy dalszej hodowli wykazują nadal przyrosty sumy alkaloidów. Na podstawie uzyskanych wyników wydawało się celowe porównanie obserwowanych zmian zawartości alkaloidów w zarodkach i liścieniach izolowanych w 4 dniu z przyrostami w tym samym czasie w nie naruszonych roślinach. Pozwoliłoby to stwierdzić,

czy dalsza obecność liścieni nie oddzielonych po okresie „organizacji" młodej rośliny wpływa jeszcze w jakikolwiek sposób na syntezę alkaloidów w zarodku. W tym celu zbadano przyrosty i zmiany zachodzące w czasie nie naruszonej rośliny, analizując oddzielnie zarodki i liścienie, przy czym za wielkość odniesienia (100%) przyjęto absolutną zawartość alkaloidów w 4-dniowej roślinie (Tabl. 6). Okazało się, że przyrost bez-

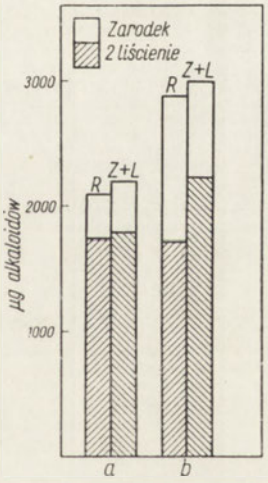
Tablica 5

Zmiany w zawartości alkaloidów w liścieniach hodowanych po oddzieleniu zarodków w 4 dniu

Dzień rozwoju po oddzieleniu zarodka	Zawartość alkaloidów (w % sumy alkaloidów w kontroli)					
	X_1	lupanina	hydroksylupanina	oksylupanina	angustyfolina	suma alkaloidów
0	0,0	42,1	48,4	0,0	9,5	100,0*
7	0,0	50,3	43,8	13,4	17,2	124,7

* Suma alkaloidów w liścieniu kontrolnym wynosiła 894,0 µg.

względnej ilości alkaloidów w roślinie jest niemal równy sumie przyrostów w izolowanych zarodkach i liścieniach. Po dalszych 7 dniach rozwoju 4-dniowej rośliny wynosił on 792 µg, podczas gdy w oddzielnie ho-



Rys. 1. Zawartość alkaloidów (µg) w jednej roślinie. *R*, nie naruszonej, *Z+L*, w izolowanych organach; a — kontrola (nie naruszona roślina lub 1 zarodek i 2 liścienie analizowane w 4 dniu rozwoju, tzn. w momencie oddzielenia liścieni od zarodka), b — kultura 7-dniowa (tj. hodowana w ciągu 7 dni od momentu analizowania kontroli)

Tablica 6

Zmiany w zawartości alkaloidów w zarodkach i liścieniach nie naruszonej rośliny

Dzień rozwoju	Zawartość alkaloidów (w % ogólnej sumy alkaloidów w 1 roślinie w 4 dniu rozwoju)												
	Zarodek						2 liścienie						ogólna suma alkaloid.
	X_1	lupanina	hydroksy-lupanina	oksy-lupanina	angusty-folina	suma alkaloid.	X_1	lupanina	hydroksy-lupanina	oksy-lupanina	angusty-folina	suma alkaloid.	
0	0,0	11,0	5,0	0,0	0,0	16,0	0,0	52,5	31,5	0,0	0,0	84,0**	100,0*
7	0,0	29,7	8,7	14,6	2,6	55,6	0,0	37,6	27,2	11,9	5,6	82,3	137,9
14	2,9	33,6	17,7	17,3	6,1	77,6	3,1	14,4	18,6	10,4	3,7	50,2	127,8

* Suma alkaloidów w roślinie kontrolnej wynosiła 2090,0 µg.

** Wartość ta obejmuje również alkaloidy wykryte w pożywce, których zawartość stanowiła 5,5% ogólnej sumy.

dowanych organach rośliny, tj. izolowanym zarodku i dwóch liścieniach, łączna suma przyrostów była równa 731—844 μg (Rys. 1).

Ponadto, z omówionego doświadczenia wynika jednak, że chociaż wielkość biosyntezy jest taka sama w nie naruszonej roślinie, jak łącznie w jej organach oddzielnie hodowanych, to przyrost sumy alkaloidów w zarodku z nie oddzielonymi liścieniami jest większy niż w izolowanym zarodku. W nie naruszonej roślinie ogólny przyrost sumy alkaloidów jest skupiony tylko w zarodku, natomiast hodowane liścienie zawierają ich nawet mniej niż 4-dniowa kontrola. Można stąd wnioskować, że obserwowany przyrost w zarodku wynika zarówno z syntezy — najprawdopodobniej niezależnej już od obecności liścieni — jak i z przemieszczania się alkaloidów syntezowanych w liścieniach.

Tablica 7

Zmiany w zawartości alkaloidów w zarodkach hodowanych z liścieniami leżącymi obok, po oddzieleniu liścieni w 4 dniu

Kultury zawierały po 10 zarodków i 2 liścienie

Dzień rozwoju	Zawartość alkaloidów w zarodkach i liścieniach (w % ogólnej sumy alkaloidów w 1 zarodku i 1/5 części liścienia)											
	Zarodek					1/5 liścienia						
	lupanina	hydroksylupanina	oksylupanina	angustyfolina	suma alkaloidów	X_1	lupanina	hydroksylupanina	oksylupanina	angustyfolina	suma alkaloidów	ogólna suma alkaloidów
0	54,9	22,0	0,0	0,0	76,9	0,0	14,3	8,8	0,0	0,0	23,1	100,0*
7	71,6	26,4	26,7	20,7	145,4	2,7	15,2	12,5	0,0	3,2	33,6	179,0
14	84,7	18,6	28,8	20,4	152,5	4,9	19,4	14,5	0,0	3,5	42,3	194,8

* Suma alkaloidów w kontroli (zarodek i przypadająca część liścienia) wynosiła 694,7 μg.

W celu przekonania się, czy możliwa jest również translokacja alkaloidów z liścieni izolowanych w 4 dniu do izolowanych zarodków, wykonano doświadczenie, w którym hodowano 10 zarodków z dwoma leżącymi obok liścieniami. W tablicy 7 podano zawartość alkaloidów w jednym zarodku i w przypadającej na niego części liścienia (tj. 1/5). W zarodkach w ten sposób hodowanych obserwuje się prawie dwukrotny przyrost sumy alkaloidów po 7 dniach w porównaniu do kontroli. Przyrost ten jest analogiczny jak w izolowanych zarodkach (Tabl. 4), a więc nie wskazuje na przemieszczanie się alkaloidów z izolowanych liścieni do izolowanych zarodków, hodowanych obok siebie.

DYSKUSJA

Zdolność poszczególnych organów młodych roślin łubinu wąskolistnego do syntezowania alkaloidów badali Mothes i Engelbrecht [1] i stwierdzili, że zdolność tę posiada tylko część nadziemna. Autorom nie udało się jednak ocenić przyrostu w części podliścieniowej, nie badali oni zupełnie oddzielonych liścieni. Już w pracy Reifera i Kleczkowskiej [2] wykazano, że izolowane po 24 godzinach: korzonki, części podliścieniowe i pączki zarodków nie są zdolne do samodzielnej syntezy alkaloidów, a tylko w obecności liścieni możliwa jest ich synteza, w tym czasie, w zarodkach.

W świetle wyników, przedstawionych w tej pracy, wydaje się, że istotnie nie część podliścieniowa, ale liścień decyduje o zdolności biosyntezy w pierwszym okresie rozwoju rośliny. Ponownie wykazano bowiem zgodnie ze wspomnianą pracą [4], że zarodek pozbawiony w 1 dniu liścieni nie posiada zdolności syntezowania, a obecność liścieni warunkuje przyrost bezwzględnej ilości alkaloidów w zarodku. Stwierdzono ponadto po raz pierwszy, że liścienie w 1 dniu izolowane syntezują alkaloidy.

Wydaje się więc, że synteza w ciągu pierwszych kilku dni rozwoju rośliny odbywa się tylko w liścieniach, a przyrosty alkaloidów w zarodku są wynikiem ich translokacji. Jak dotychczas, nie można jednak wykluczyć także możliwości wpływu obecności liścieni w roślinie na to, że już w 1-dniowym zarodku mogłaby w pewnym stopniu dokonywać się synteza. Natomiast nie podlega żadnej wątpliwości fakt, że zarodki, które w 4 dniu rozwoju pozbawiono liścieni, w ciągu dalszej hodowli mogą samodzielnie syntezować alkaloidy. Również liścienie izolowane po tym samym okresie czasu wykazują nadal zdolność do syntezy. Z wyników uzyskanych dla nie naruszonych roślin okazało się, że zdolność do samodzielnej syntezy w zarodku, po upływie początkowego okresu, w którym muszą one pozostawać w łączności z liścieniami (tj. do 4 dnia rozwoju — w warunkach doświadczeń), jest już później niezależna od obecności liścieni.

Obserwowana zdolność do samodzielnej syntezy alkaloidów, przez zarodki izolowane w 4 dniu rowoju, jest zgodna z wnioskiem I części pracy [6], że synteza tych związków następuje dopiero po okresie „organizacji" młodej rośliny (od 0—4 dni).

Zmiany składu alkaloidów, obserwowane poprzednio w nie naruszonych roślinach [6], tj. przyrost lupaniny oraz pojawienie się oksylupaniny i angustyfoliny, wykazano obecnie również w oddzielonych liścieniach i zdolnych do syntezy zarodkach. W przeciwieństwie do tych obserwacji, zarodki pozbawione liścieni po 1 dniu moczenia nasion nie są zdolne do syntezy angustyfoliny i oksylupaniny, a obserwowanemu przyrostowi lupaniny odpowiada spadek hydroksylupaniny.

STRESZCZENIE

1. Zbadano zdolność do syntezowania alkaloidów w izolowanych zarodkach i liścieniach łubinu wąskolistnego w kulturach jałowych.

2. Wykazano, zgodnie z wcześniejszą pracą Reifera i Kleczkowskiej [2], że zarodek w 1 dniu izolowany nie może syntezować alkaloidów.

3. Zarodek izolowany w 4 dniu syntezuje alkaloidy w okresie dalszego rozwoju. Zdolność tę wykazują również liścienie izolowane w tym samym czasie.

4. Przyrost bezwzględnej ilości alkaloidów w roślinie jest zbliżony do sumy przyrostów w izolowanych zarodku i liścieniach.

5. Zdolność zarodka do syntezy alkaloidów od 4 dnia rozwoju nie jest związana z obecnością liścieni.

6. Ogólnie przyrost sumy alkaloidów jest spowodowany przyrostem lupaniny oraz pojawieniem się angustyfoliny i oksylupaniny.

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BIOSYNTHESIS AND CHANGES OF ALKALOID CONTENT IN THE BLUE LUPINE *(L. ANGUSTIFOLIUS)*

II. BIOSYNTHESIS OF ALKALOIDS IN ISOLATED GERMS AND COTYLEDONS

Summary

1. The alkaloid synthesis in isolated germs and cotyledons in sterile cultures of the blue lupine has been investigated.

2. In agreement with former work by Reifer and Kleczkowska, it was shown that germs separated after the day and then grown in sterile cultures have no ability to synthesise alkaloids.

3. On the other hand, germs separated after the fourth day show a distinct ability of alkaloid synthesis on further growth.

4. The increase of the absolute amounts of alkaloids in the whole plant is equal to the increases in the germ and cotyledons grown separately.

5. The ability of the 4-day old germ to synthesise alkaloids is independent of the presence of cotyledons.

6. The increase of the sum of total alkaloids is caused by an increase of lupanine and the appearance of angustifoline and oxylupanine.

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THE URIC ACID RIBOSIDE IN *SPHINGIDAE* MOTHS*

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Uric acid bound to ribose was first detected in 1922 by Benedict and co-workers [3] in ox erythrocytes and later by Falconner and Gulland in 1939 [4] in ox liver. The occurrence of specific enzymes splitting the uric acid riboside might indicate its probably large spread distribution in living organisms. In many mammalian tissues, for instance, as well as in pigeons [9], an enzyme was found which split the uric acid riboside to give uric acid and ribose-1-phosphate, acting thus likewise nucleoside phosphorylase. Another kind of enzyme, corresponding to nucleosidase and splitting the riboside into uric acid and ribose, was found in *Lactobacillus pentosus* [8].

In the present work the uric acid riboside was found in diapausing pupae of *Sphinx pinastri* and *Celerio euphorbiae,* both species belonging to the family of *Sphingidae.* It was possible to find this riboside only when some methodical modifications had been introduced to the procedure of removing protein. The uric acid riboside is strongly adsorbed on protein and its loss on acid precipitation of protein may reach up 50 per cent [6]. Therefore, we have removed protein by heat denaturation in alkaline solution instead of acid solution applied by Benedict *et al.* [3].

Fat-bodies of diapausing pupae were homogenized in Potter-Elvehjem homogenizer with 10-fold volume of 0.2 M-borate buffer ($NaBO_3$, NaOH), pH 10. Homogenate was boiled for 1—2 minutes and the precipitated protein centrifuged off. The liquid was cautiously decanted to separate the fatty layer. To remove the residue of non-precipitated protein, the supernatant was shaked several times with chloroform, according to Sevag *et al.* [12], and centrifuged each time, the water phase being decanted from over the layer of chloroform and that of precipitated protein.

* Paper contributed by the authors to the issue dedicated to Professor Dr. Włodzimierz Mozołowski.

The protein-free liquid was acidified with sulphuric acid to pH 3—4 and allowed to stay overnight in refrigidator to enable a partial precipitation of uric acid, which is very abundant in fat-body and renders difficult the chromatographic identification of riboside. The uric acid crystals being filtered off, the filtrate was subjected to ascending chromatography on Whatman No. 3 paper in 60% water solution of *n*-propanol, as applied by Leone and Guerritore [10] to separate the riboside from the uric acid. Chromatogram was then dried and two spots were visible in ultraviolet light, their R_F values being 0.42 (spot I) and 0.52 (spot II). Leone and Guerritore had found R_F values of 0.47 and 0.57 for uric acid and its riboside, respectively, when using the descending technique on Whatman No. 1 paper. Thus spots I and II might correspond to these compounds, since the distance between the spots remained the same; the lower values of R_F might be due to somewhat different conditions of chromatography, as well as to the rather high concentrations of salts in the chromatographed solution.

To begin with, the solution obtained from ox erythrocytes was examined chromatographically with the technique described above, since the uric acid riboside had been first found in this material. One liter of ox blood was shaked with glass beads to remove fibrin, centrifuged, and erythrocytes added with equal volume of distilled water and 10 ml. of chloroform. Hemolyzed erythrocytes were then treated with twice that volume of 0.2 M-borate buffer and protein removed in the same manner as from the pupae homogenate. The dark sediment precipitated on acidification with sulphuric acid was removed by centrifugation. The supernatant was then concentrated on boiling water bath to the final volume of 50 ml., since ox erythrocytes are known to contain rather small amounts of the riboside. Some sediment further formed during this evaporation was withdrawn and the liquid was subjected to chromatography. Two spots visible in ultraviolet light were found. The R_F values for these spots were analogous to those found when the pupal fat-body homogenate was examined.

The phosphotungstic acid reaction for uric acid [1] was performed on chromatograms of the two solutions, as well as in water eluates deriving from the excised spots both of the ox blood and of fat-body of the pupa. Spot I gave a positive reaction while spot II did not, neither in eluates nor on paper. When the eluate deriving from spot II was hydrolysed for several hours in 0.1 N-HCl at 100° — positive reaction for uric acid was stated. These data indicate that the spot II corresponds to the uric acid riboside, which is known to produce a positive reaction with phosphotungstic acid only after hydrolysis [3].

The orcinol reaction [11] was performed in water eluates, deriving from the pupae chromatogram to detect the presence of sugar bound to uric acid. The positive reaction for pentose was obtained in eluates from the spot II only. This eluate was hydrolysed with 0.1 N-HCl, then chromatographed according to Giri and Nigam [5] in the following solvent system: acetone - *n*-butanol - water (7:2:1). The presence of ribose was stated on the chromatogram developed with triphenyltetrazolium chloride [13].

To ascertain whether the investigated compound is not a ribotide, chromatogram of the protein-free homogenate was tested for the presence of phosphate [7], but no blue colour was produced on chromatogram in the place where the spot II was visible in ultraviolet light. In the eluate also neither free or bound phosphate was detected.

The results obtained by chromatography, as well as positive colorimetric reactions for uric acid and ribose indicate that the compound giving rise to the spot II is the uric acid riboside. We have found this riboside in the diapausing pupae of *Sphinx pinastri* and *Celerio euphorbiae* in fat-body, but not in the haemolymph. It is noteworthy to mention that the concentration of the uric acid riboside in pupae is rather high. The chromatograms allow to estimate its content in fat-body as surpassing 10 times, at least that found in ox erythrocytes.

Falconner and Gulland in 1939 [4] presented the absorption spectrum of uric acid riboside in distilled water with two maxima resembling the uric acid spectrum. Carter and Potter in 1952 [2] reported that they had found another absorption spectrum, but they did not present their own data. Therefore, we have investigated spectra of uric acid and its riboside derived from the spots I and II on chromatograms prepared both from fat-body homogenate and from ox blood. The spots were excised and eluted with 0.1 N-NaOH, with hot distilled water, or with 0.1 N-HCl, then absorption was measured in eluates in Unicam spectrophotometer at 210 to 320 mμ. Figures 1 and 2 show the absorption curves yielded by uric acid and its riboside derived from fat-bodies of *Sphinx pinastri* pupae. The analogous curves have been found when these two compounds of the *Celerio euphorbiae* fat-bodies and those of ox blood were examined. As it can be seen in Fig. 1, the uric acid and its riboside in 0.1 N-NaOH yield spectra similar one to another and characterized by one maximum at 295 mμ corresponding to those of the standard uric acid solution. The spectrum of the 0.1 N-HCl eluate (Fig. 1) differs from the former: two maxima are found for the uric acid, such at those given by standard uric acid in acid solution, whereas one maximum only is detected for the

riboside, and only a slight deformation of the curve at 220—235 mμ is marking the place of the other one. The water eluates yield spectra (Fig. 2) analogous to those found in the acid ones but no deformation like that mentioned above is visible in curve of the uric acid riboside. The position of maximum, as well as that of minimum, is shifted somewhat towards shorter waves when the alkaline medium is substituted by an acid one.

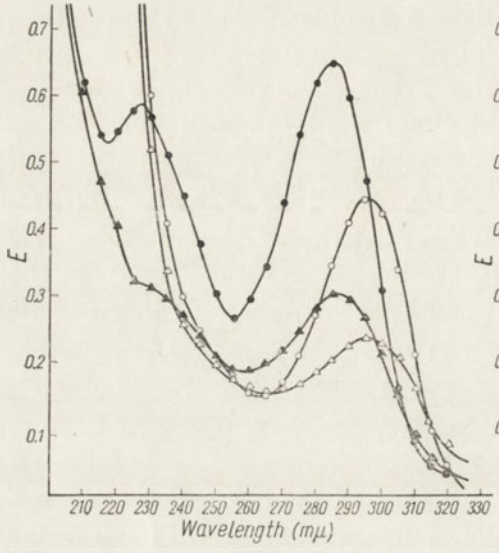

Fig. 1

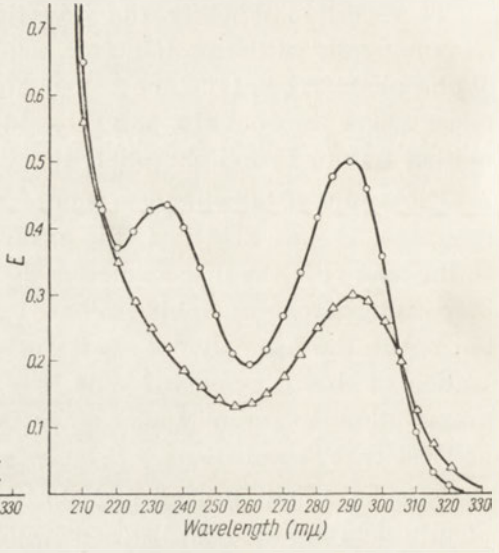

Fig. 2

Fig. 1. The absorption curve of uric acid and its riboside in 0.1 N-NaOh and in 0.1 N-HCl. Eluates derived from the spots I (uric acid) and II (uric acid riboside) of the chromatograms of *Sphinx pinastri* pupae fat-bodies. (o), uric acid in 0.1 N-NaOH; (●), uric acid in 0.1 N-HCl; (△), uric acid riboside in 0.1 N-NaOH; (▲), uric acid riboside in 0.1 N-HCl

Fig. 2. The absorption curve of uric acid and its riboside in distilled water. Eluates derived from the spots I and II of chromatograms of the *Sphinx pinastri* pupae fat-bodies. (o), uric acid (spot I); (△), uric acid riboside (spot II).

The occurrence of the uric acid riboside in the pupal fat-body in rather high concentration rises the question of its possible biological significance, but as yet we can only express some suggestions. This compound migth play some role in the transfer of uric acid from fat-body to Malpighian tubules and to posterior part of intestine since it is more soluble than uric acid is. It might be also considered as an probable intermediate in the process of transformation of purine ring into the pteridine one.

SUMMARY

The uric acid riboside was found in diapausing pupae of *Sphinx pinastri* and *Celerio euphorbiae*, its extraction being carried out by means of borate buffer, pH 10. It was separated chromatographically and colour tests for the presence of uric acid and ribose were performed. The spectrum of this compound was examined in ultraviolet light and the results were compared to those found for uric acid riboside obtained from ox blood.

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RYBOZYD KWASU MOCZOWEGO U MOTYLI Z RODZINY ZAWISAKÓW

Streszczenie

Znaleziono rybozyd kwasu moczowego u zimujących poczwarek *Sphinx pinastri* i *Celerio euphorbiae*, stosując ekstrakcję buforem boranowym o pH 10. Wydzielono go chromatograficznie, przeprowadzono reakcje barwne na kwas moczowy i rybozę. Zbadano widmo w nadfiolecie i porównano z danymi dla widma rybozydu kwasu moczowego, uzyskanego z krwi wołowej.

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INVESTIGATIONS ON HISTOCHEMICAL LOCALIZATION OF NUCLEASE ENZYMES

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The localization in living cells of the sites of the various enzymes exhibiting activity towards nucleic acids is a problem of considerable importance in relation to nucleic acid metabolism and has formed the subject of a number of investigations, based largely on cell fractionation techniques [2, 8, 14, 15, 36, 37, 50, 51, 54, 55, 63]. However, histochemical methods frequently offer distinct advantages with regard to precision of localization as compared to fractionation techniques, as well as the greater ease of conducting routine studies on the large numbers of samples which must be handled in most investigations.

One of the earlier methods for the histochemical localization of nuclease enzymes was that of Marshall [39], based on the use of fluorescein labelled antibodies to ribonuclease and deoxyribonuclease. The procedures more recently described by Daoust [9, 10] and Daoust & Amano [11] for deoxyribonuclease and ribonuclease, respectively, are applicable mainly to the gross localization of these enzymes in tissue sections. Several publications [29, 30, 45] have dealt with the effects of various fixatives on ribonuclease activity, presumably as a preliminary to preparation of the ground for the development of histochemical methods for this enzyme in fixed material.

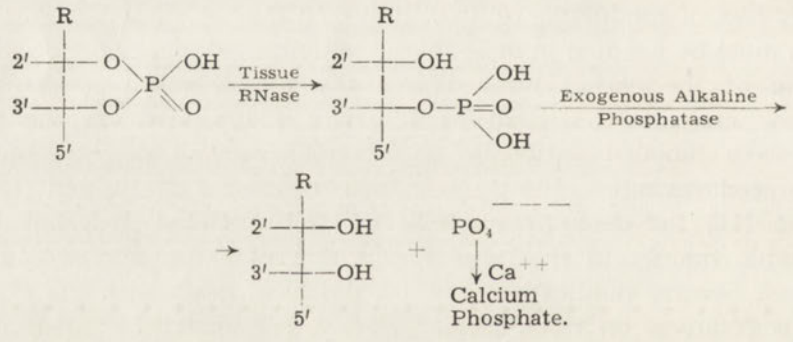
While this problem may have appeared to be a relatively straightforward, albeit not a simple, one only a few years ago, it has now become considerably more complex as a result of the discovery of a variety of RNases, DNases and PDases [1], exhibiting varying specificities towards RNA and DNA [6, 7, 12, 13, 21, 22, 23, 25, 26, 31, 38, 46, 49, 52, 65, 67, 68].

[1] The following abbreviations will be used in this text: RNase, ribonuclease; DNase, deoxyribonuclease; PDase, phosphodiesterase; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; Up, uridine-2′(3′)-phosphate; Cp, cytidine-2′(3′)-phosphate;

At least for some of the tissue RNases there exist several simple substrates, viz. the nucleoside 2′ : 3′ cyclic phosphates and nucleoside 2′(3′) alkyl phosphates. The development, in our laboratory, of a simple procedure for the quantitative conversion of Up, Cp and Ap to the corresponding cyclic phosphates [61] made available in highly purified form several substrates for RNase-like enzymes and prompted us to examine the possibilities of the direct histochemical localization of these latter. Although our original objective has not been attained, the interest in this problem is such that the findings may prove useful to future investigators in this field.

During the course of this investigation a publication appeared by Aronson *et al.* [3] in which a similar approach was utilized for the localization of acid DNase, based on the use of DNA as substrate and with acid phosphatase added to the incubation medium to liberate terminal phosphate groups from the enzymatically hydrolyzed DNA. We have therefore also examined the procedure of the foregoing authors in relation to the difficulties we have ourselves encountered with our attempted technique for RNase.

The procedure devised is essentially a two-stage enzymatic reaction, the second of which is based on the Gomori technique for alkaline phosphatase, as follows (where R is a purine or pyrimidine):



The incubation medium consists of a solution containing an appropriate buffer, a cyclic nucleoside phosphate as substrate, purified alkaline phosphatase and a soluble calcium salt. Hydrolysis of the nucleoside cyclic phosphate at the site of RNase activity should result in the appearance of a nucleoside 2′ (or 3′) phosphate [2]. This is then acted upon by the added alkaline phosphatase, the resultant liberated phosphate ions being trapped

Ap, adenosine-2′(3′)-phosphate; Up!, uridine-2′ : 3′-phosphate; Cp!, cytidine-2′ : 3′--phosphate; Ap!, adenosine-2′ : 3′-phosphate.

[2] There are some RNases which will not attack nucleoside cyclic phosphates [46, 48].

by the calcium to form the usual precipitate of calcium phosphate. The course of the reaction may be followed quantitatively by the use of ^{45}Ca or ^{32}P-labelled nucleoside cyclic phosphates [56, 59, 60], and localization by staining, according to the usual Gomori procedure [18].

At least two major difficulties might be foreseen with such a procedure: (a) nucleoside cyclic phosphates are hydrolyzed only relatively slowly by RNases, so that unduly long incubation times may be required; (b) the use of an enzyme in the second stage of the reaction is not the best practice since free penetration of such a large molecule into the tissue section may be hindered. A third conceivable obstacle is the possibility that exogenous phosphatase in the incubation medium might release inorganic P from organic phosphates in the section; this factor was, however, found experimentally to be of no importance.

MATERIALS AND METHODS

Attempts at histochemical localization were limited to those RNases active at alkaline pH. Furthermore the pH of the incubation medium was somewhat higher than that corresponding to optimum RNase activity; this was considered necessary in order to ensure a good capture reaction by providing optimal conditions for alkaline phosphatase activity as well as for minimum solubility of the final calcium phosphate precipitate [28].

The incubation medium consisted of 0.028 M-veronal-acetate buffer pH 9; 0.01 M-$CaCl_2$; 0.0016 M-$MgSO_4$; 0.01 M-substrate (nucleoside cyclic phosphate); and purified alkaline phosphatase (Sigma) at a concentration of 0.2—0.3 mg./ml. Although the phosphatase preparation was found to be quite active against calcium (bis) *p*-nitrophenyl (phosphate)$_2$ and RNA, suggestive of the presence of some phosphodiesterase activity, its activity vs. nucleoside cyclic phosphates was practically negligible.

Because of the limited amounts of substrate available, the incubation procedure was similar to that previously described for use with labelled materials and referred to as the "drop" method [59, 60]. 10 μ sections of various rat tissues, fixed in chilled 80° ethanol and embedded in paraffin, were used throughout. Sections mounted on glass slides were placed in warm Petri dishes saturated with water vapour and about 30 μl. of the incubation medium deposited on each section. After one hour at 37°C. incubation was interrupted by immersion of the slide in 40% acetone [60]. The slide was then dried in a stream of cold air and submitted to further incubation. Usually three hours incubation was sufficient for tissues with high enzymatic activity. The precipitate formed was rendered visible by cobalt nitrate and ammonium sulfide treatment according to Gomori [18]. In initial experiments labelled calcium was employed in order to deter-

mine whether the expected reaction was taking place, without having to resort to staining [60].

The incubation medium was prepared fresh immediately prior to an experiment and was centrifuged each hour to remove the slight cloudiness formed on standing. Clouding of the solution was found to be due to the hydrolytic effect of calcium (or of lead in acid medium) on the nucleoside cyclic phosphates, leading to the formation of nucleoside-2′(3′)-phosphates. This, of course, also occurred during incubation, thus competing to some extent with the action of ribonuclease. Equally serious, however, is the possibility that in sections rich in endogenous phosphatase, the latter might act on these phosphates and lead to the formation of non-specific localization of calcium phosphate.

In view of the foregoing it was necessary to introduce adequate controls which, in addition to those in which the incubation medium was deficient in either substrate or phosphatase, included also sections inactivated by heating for 30 mins. in water or buffer (pH 9), or by ultraviolet irradiation.

The histochemical results were also controlled by paper chromatography of the constituents of the incubation medium, using ascending chromatography with *iso*propanol-ammonia-water (23 : 7 : 3, v/v/v) and descending chromatography with butanol-acetic acid-water (4 : 1 : 5, v/v/v) on Whatman paper No. 1.

RNase appearing in the incubation medium, as a result of diffusion from the tissue section, was measured according to the method of McCarty [41].

RESULTS AND DISCUSSION

Unless otherwise indicated the results refer to those obtained with the use of Up! and Cp! as substrates. In the absence of substrate all tissues examined gave a negative reaction.

The pancreas, spleen, kidney and duodenum gave net positive reactions (Figs. 1, 2, 3) while liver exhibited only a weak precipitate. The precipitates were localized mainly in the nuclei and nucleoli; in addition the kindney and duodenum exhibited a cytoplasmic reaction at the sites of endogenous phosphatase (Figs. 2, 3). The intensity of staining was found to correspond to the nuclease activity of the various tissues as estimated by the rate of hydrolysis of a solution of Cp! by tissue sections in the absence of calcium ions, and measured by the rate of disappearance of Cp! on paper chromatograms. The relative activities of the tissue RNases determined in this way were also in agreement with those estimated on tissue homogenates [4, 16].

Heat inactivated sections exhibited only a faint, uniform precipitate over the entire section, with no characteristic localization. Curiously enough, even prolonged irradiation (24 hours) with a high pressure mercury lamp was insufficient to completely remove enzyme activity (even in 6 μsections) despite the high quantum yield for ultraviolet inactivation of purified RNase [57, 58] and the ready inactivation of alkaline phosphatase under similar conditions [62].

Incubation in media deficient in exogenous phosphatase was found to give positive results only in sections rich in endogenous phosphatase (kidney and duodenum), the intensity and localization of the precipitate corresponding to that for the endogenous enzyme in the section. Tissues comparatively poor in alkaline phosphatase, such as pancreas and liver were negative.

This latter observation, together with the fact that apparent localization of RNase was mainly in nuclei and nucleoli, led to the suspicion that the tissue RNase might be diffusing into the medium during incubation [3]. Lagerstedt [35] has reported indirect evidence for such diffusion, based on the observation that nucleotide-like material is released from Carnoy-fixed tissue sections during incubation in McIlwaine buffer, with an accompanying loss of cytoplasmic basophilia. It consequently seemed appropriate to introduce as supplementary controls sections which had been previously exposed to a buffer solution, thus allowing enzyme diffusion to occur prior to incubation proper and leading, perhaps, to more precise localization of the desmoenzyme [42, 43]. Examination of such "preincubated" sections for total RNase content showed that, although they retained some enzyme activity, more enzyme had been removed by 1 hour preincubation than by 24 hours ultraviolet irradiation. Examination of the buffer in which the sections had been preincubated showed the presence of considerable RNase, but no significant phosphatase, activity. Subsequent examination of these preincubated sections revealed them to be markedly less positive, but with similar localization.

Leaching out of the enzyme was found to occur both at acid and alkaline pH and was found to be rapid for both acid and alkaline RNases of the pancreas, spleen, kidney and liver. The rate of diffusion was

[3] If appreciable diffusion of enzyme from a section were taking place, this would be rapidly obvious with our "drop" method of incubation, since the liberated enzyme is concentrated in a small volume. In this respect our incubation procedure is definitely inferior to those normally used, where enzyme diffusing from a section is diluted considerably by the relatively large incubation volumes employed. On the other hand the use of a small volume of incubation medium is much more effective for determining the extent to which such diffusion is occurring.

generally greater in alkaline than in acid or neutral medium and was such that a pancreas section lost up to 75% of its activity against RNA to the medium in 15 minutes. It therefore seemed most likely that the positive reactions observed were due to the enzyme diffusing into the incubation medium. This was confirmed by incubating „preincubated" sections (which normally are only slightly positive) in a medium the stock buffer of which was that used for preincubation. Under these conditions preincubated sections were found to be strongly positive (Fig. 4). The same result was achieved by addition of crystalline RNase to a normal incubation medium.

Additional evidence that the positive reactions observed under normal incubation conditions were due mainly to enzyme diffusion was furnished by the following experiments: (a) when a preincubated section was incubated in a normal medium together with a normal section, the latter exhibited a weaker positive reaction and the former an enhanced one; (b) when a normal section was incubated in progressively larger volumes of normal incubation medium (20 to 800 μl) the resultant staining intensity became progressively weaker; (c) liver sections, normally exhibiting little nuclease activity (cf. [4, 16]), show an intensified nuclear reaction when incubated together with a pancreas section.

It therefore follows that the positive reactions observed are due, to a considerable extent, to the formation from the substrate of reaction products which exhibit a strong affinity for nuclei [19, 27, 44, 66]. Now since ultraviolet irradiated sections contain more enzyme than pre-incubated sections, they should give a reaction intermediate to that between normal and preincubated sections. In addition, heat inactivated sections, which contain no nuclease activity, would be expected to show a positive reaction on prolonged incubation as a result of partial hydrolysis of the substrate by calcium (see above). This is found not to be the case. Ultraviolet irradiated sections accumulated less precipitate than preincubated ones despite their higher nuclease content, while heat inactivated sections did not show a nuclear precipitate even after extensive incubation. In fact, preincubated sections incubated with added enzyme gave a slightly more positive cytoplasmic reaction than normal sections. The observed positive reactions must therefore be due to modifications in affinity of the tissues towards the final calcium phosphate precipitate as a result of heating, ultraviolet irradiation and, to some extent, of preincubation.

That such is indeed the case was shown by incubating normal, preincubated, ultraviolet-treated and heat-inactivated sections of liver and pancreas in a medium containing either (a) Cp, alkaline phosphatase and

calcium, or (b) Cp!, RNase, alkaline phosphatase and calcium. The heat inactivated sections exhibited a moderate positive reaction but with no noticeable localization; ultraviolet irradiated sections showed a markedly decreased positive reaction at the nuclei and nucleoli; while the changes in the preincubated sections were slight but with a definite tendency towards a decreased positive reaction at the nuclei as compared with the cytoplasm.

The validity of heat inactivated sections as controls therefore appears to be questionable (cf. Bartelmez & Bensley [5], and Pearse [47]) since heat treatment has apparently done more to the section than merely remove enzyme activity. To a lesser extent the use of ultraviolet inactivated sections is also open to question.

Similar difficulties were encountered in the use of Ap! as substrate. The enzyme active against Ap!, most abundant in the spleen and less so in the kidney and pancreas, was also found to diffuse rapidly from all these sections. Preincubated sections exhibited a less intense reaction and, in general, similar localization artifacts were encountered as those reported above for pyrimidine nucleoside cyclic phosphates.

The foregoing procedure for ribonuclease-like enzymes is therefore unsuitable principally because of the high rate of diffusion of the enzymes from the sections. The hydrolytic effect of calcium ions on the substrate, while bothersome, could be readily circumvented if some means of preventing escape of the enzyme were devised.

We have made several attempts to use yeast RNA as substrate in place of nucleoside cyclic phosphates, since, although it is less specific, it is hydrolyzed much more rapidly by RNases, so that shorter incubation periods might be used with resultant reduced diffusion of the enzyme. No positive reaction could, however, be obtained for incubation periods of up to 15 minutes; and the localization obtained after 1 hour's incubation of a pancreatic section was similar to that resulting from the use of Cp! as substrate, the RNase having largely diffused out of the section during this period.

The diffusion of various enzymes from tissue sections, prior to and following fixation, has been extensively investigated by Hannibal & Nachlas [24], who found the largest rate of diffusion from fixed tissues for esterases and β-glucoronidase. Even acid phosphatases showed appreciable diffusion, in agreement with Gomori [20] who reported that 30% and 50% of the acid phophatase could be extracted within 6 hours from formalin-fixed liver and prostate sections, respectively. The use of fixatives such as formalin was found by Nachlas *et al.* [42] to retard the

diffusion of some enzymes, but the RNase of pancreas sections appears to be completely inactivated by this reagent [30].

It is therefore clear from the above that the development of a suitable histochemical procedure for ribonuclease enzymes requires the preliminary establishment of some type of fixation procedure which will result in the retention by tissue sections of at least a portion of the enzyme. The results of Scarpelli & Pearse [53] suggest that enzyme diffusion may be limited by using suitable osmotic and chemical protection of fresh frozen sections; if such were to be the case it still remains to be seen whether the exogenous alkaline phosphatase used in the above procedure would be able to penetrate the intact particle membranes of the material under study. The procedures developed by Daoust [9, 10] and Daoust & Amano [11] for the gross localization of DNase and RNase are probably largely free from errors due to enzyme diffusion thanks to the short incubation periods employed and the use of a gelatine medium for presenting the substrate to the tissue section.

Deoxyribonuclease

We originally made no attempts to apply the above procedure to DNase-like enzymes because of the fact that well defined low-molecular weight substrates are not available for these enzymes, while their natural substrate has a molecular weight of the order of one to several million which might be expected to render its accessibility to DNase in a tissue section at best somewhat tenuous. The publication, during the course of the above work, of the procedure of Aronson *et al.* [3] for DNase has now been followed by its application to the estimation of the effect of irradiation on DNase II in animal tissues by Aldridge *et al.* [1].

In the original procedure [3], fresh frozen sections were fixed in acetone-formalin-water (50 : 10 : 40), which was found to give an increased staining intensity and improved cellular detail as compared to fixation with 50% acetone. In the application of this technique to tissues from irradiated animals, however, the fixation procedure was modified as follows: neutral formol-saline fixation of tissue blocks, washing in acetate buffer, clearing and dehydrating by passage through absolute acetone, benzene-acetone and benzene, and paraffin embedding [1].

In view of the known similarities in intracellular localization of RNases and DNases [8, 15, 54, 63, 64], it appeared of interest to us to examine several aspects of the above techniques, using the fixation procedure of Aldridge *et al.* [1]. Bearing in mind our own findings for RNase, we initially undertook an examination of the diffusibility, if any, of DNase from tissue sections. DNase II was assayed according to Allfrey &

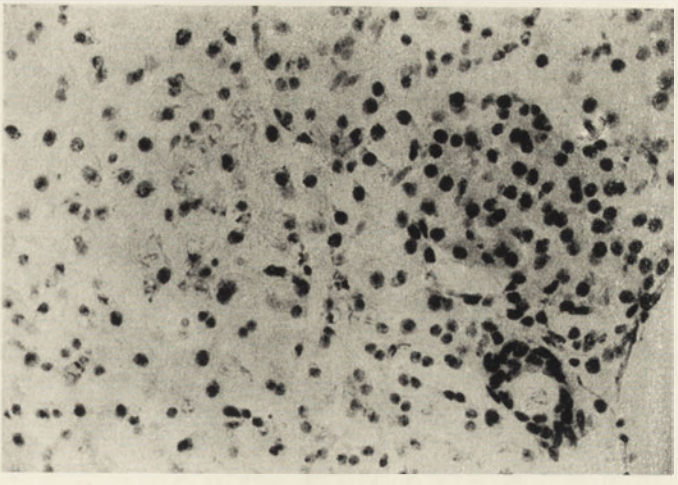


Fig. 1. Rat pancreas incubated with Cp!, showing nuclear and nucleolar staining, particularly heavy in the Islet of Langerhans (×320)

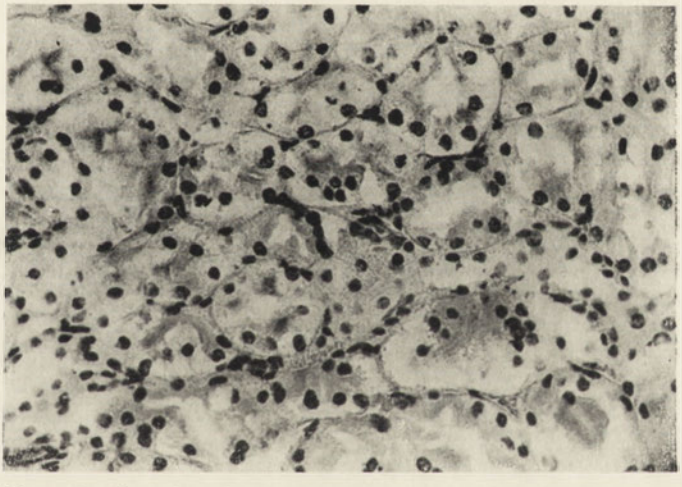


Fig. 2. Rat kidney incubated with Cp! showing heavy nuclear and nucleolar staining; note staining of brush border typical for phosphomonoesterase (×320)

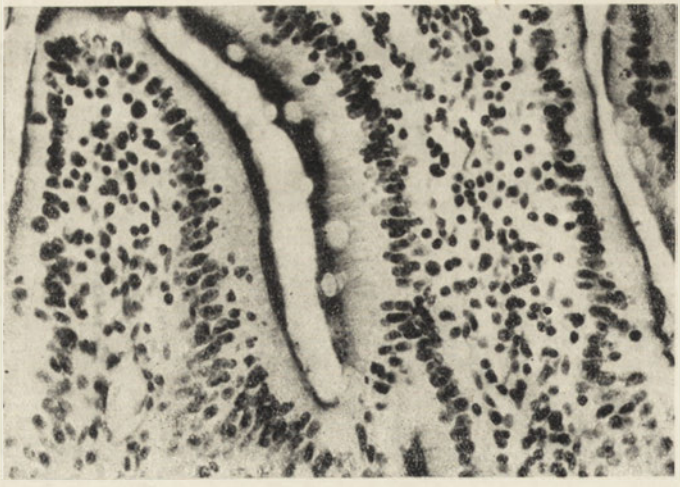


Fig. 3. Rat duodenum incubated with Up!; heavy staining of nuclei and nucleoli of epithelial cells and lamina propria; and exhibiting also staining of brush border typical for phosphatase (× 320)

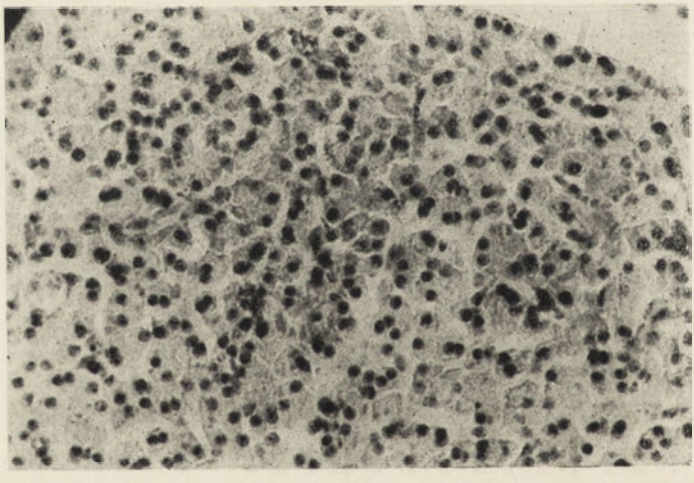


Fig. 4. Preincubated rat pancreas submitted to incubation in medium containing buffer used in "preincubation" and exhibiting nuclear and nucleolar stainnig of acinar cells as well as increased diffused staining of the cytoplasm (×320)

Table 1

Effect of processing according to Aldridge et al. [1] on rat spleen DNase II activity

Stage of processing	Activity remaining (per cent of initial activity)	Number of determinations
Fresh tissue	100	6
Following neutral formol-saline fixation, 2 hrs. at 4° C.	70	4
Following rinse in 0.05 M-acetate buffer, pH 5, 4 hrs. at 4°C.	58	6
Following acetone fixation, 4 hrs. at 4°C.	45	2
Following acetone-benzene (1:1) 4 hrs. at 4°C.	35	2
Following benzene 24 hrs., room temperature	13	2
Following paraffin embedding and removal in benzene	< 5	6

Mirsky [2] which allows of the determination of the enzyme in as little as 0.5 mg. rat spleen tissue. However, in our hands, formalin-acetone fixation according to Aldridge *et al.* [1] was found to destroy over 95% of the DNase activity originally present in fresh tissue. On the other hand, fixation in cold acetone alone, followed by paraffin embedding, removes only 45% of the original activity. Table 1 shows that it is not the formalin which causes the destruction of DNase activity since formalin fixation alone followed by rinsing in buffer removes only 40% of the activity, the remainder vanishing during subsequent fixation and embedding. Since the latter steps involve only cold acetone fixation and paraffin embedding which, when applied without formalin was found to remove only 45% of the activity, one would expect at least 20% of the enzyme to be left intact in the double fixed tissue sections in place of the less than 5% actually observed.

It is therefore of interest to note that Aronson *et al.* [3] actually assayed biochemically the amount of enzyme left following fixation and found this to be about 30%, which is roughly in agreement with our results. Aldridge *et al.* [1] do not provide any data as to the effect of their method of fixation on enzymatic activity.

As a result of the above we were able to estimate the relationship between the lyo and desmo components of DNase II only in acetone fixed material. Following deparaffinization in benzene and air-drying, sections were immersed in 0.05 M-acetate buffer pH 5 for 1 hour at 37°, then removed from the slides and homogenized according to Hannibal &

Nachlas [24]. One hour "incubation" in buffer was found to remove about 60% of the enzyme (Fig. 5). Following 24 hours exposure to the buffer solution, less than 5% of the original activity is retained by the sections. That this loss in activity is actually due to diffusion, and not inactivation, was demonstrated by estimation of the DNase activity appearing in the "incubation" medium (Fig. 5) [4]. The enzyme activity diffusing into the buffer during 3 hours "incubation" can be seen from Fig. 5 to equal 104% of the original activity found in a homogenate of the section. Following 24 hours "incubation" the enzyme activity which has diffused is equal to about 200% of that originally present in the sections, a finding suggestive of the presence of a non-diffusing DNase

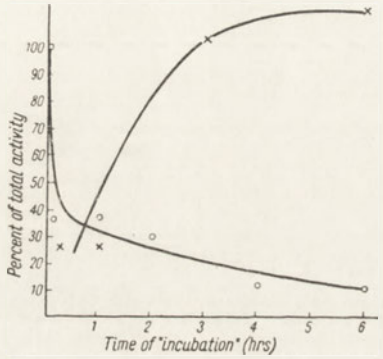


Fig. 5. Showing deoxyribonuclease diffusion out of acetone-fixed, paraffin-embedded, rat spleen sections during "preincubation" in 0.05M-acetate buffer pH 5 at 37°C: (o), activity remaining in sections, (x), activity of buffer solution

II inhibitor in the spleen tissue. Feinstein [17] has reported the presence of DNase I inhibitors in spleen and other tissues and Kurnick *et al.* [33, 34] have postulated the existence of a DNase II inhibitor in the spleen and thymus.

The effect of enzyme diffusion was also observed histochemically by "dropwise" incubation of acetone fixed kidney sections with 20 µl. of the incubation medium used by Aronson *et al.* [3] and Aldridge *et al.* [1]. A positive reaction was obtained within 3 hours, whereas a "preincubated" section gave a much weaker reaction. If, however, the preincubation

[4] DNase diffusion into "incubation medium" was determined by extraction of about 40 sections (forming a multilayer on the bottom of a test tube) with appropriate buffer solution. Because of the packing of the sections diffusion of the enzyme was slower than from individual sections mounted on a slide; this probably explains why, in Fig. 5, the enzyme activity appearing in the buffer medium is initially lower than that lost from individual sections on a slide.

buffer was used as part of the complete incubation medium on the preincubated section, the latter exhibited a more intense reaction with similar localization. The same result could be achieved by addition to the incubation medium of crystalline DNase I, which still exhibits some activity at pH 5.

Some consideration must therefore be given to the source of the positive reactions obtained (a) by Aldridge *et al.* [1] on formalin-acetone fixed sections which, under our conditions, practically completely removes DNase II activity, and (b) by Aronson *et al.* [3] on control sections devoid of exogenous acid phosphatase, where increase of the incubation time leads to a positive reaction equal to that obtained with the complete incubation medium. The latter phenomenon is assumed by Aldridge *et al.* [1] to be due to the action of „stable intracellular nucleotidases". Since endogenous acid phosphatase is known to exhibit appreciable diffusion [24, 43], and incubation times of up to 18 hours were used, this seems to be a reasonable assumption. If such is indeed the case, it suggests the possibility that in control sections both the endogenous DNase II and acid phosphatase, while diffusing from the section, meet near the surface and act on the substrate with the resultant formation of a positive reaction not representative of the true localization of either enzyme. Aronson *et al.* [3] propose an alternative explanation, viz. that the method is applicable when the localization of both enzymes in a tissue section is the same. In any event the localization obtained is contrary to the predominantly non-nuclear localization of DNase from cell fractionation investigations [2, 8, 15, 54, 64].

We have also compared the behaviour of formalin-acetone fixed spleen sections and acetone-fixed material. Incubation was carried out by immersing of individual sections on small slides in 0.5 ml. incubation medium. Acetone-fixed sections were found to exhibit a net positive reaction following 2 hours incubation, initially at the centres of the lymphoid nodules, and increasing with time until after 5 hours there was marked staining of the red pulp; but these results were not consistently reproducible. Incubation of formalin-acetone sections for 3—5 hours gave localization patterns similar to those of Aldridge *et al.* [1], but again not always reproducible. Reproducible staining patterns were obtained on sections fixed by either procedure only following 18 hours incubation; under these circumstances the acetone-fixed sections stained 3—4 times as intensely as the formalin-acetone fixed material, in agreement with the much higher DNase content, following fixation, of the former.

The behaviour of heat-inactivated sections was also found to be dependant on the type of fixation used. Acetone fixed inactivated sections

were practically negative even after 18 hours incubation; whereas the formalin-acetone fixed material exhibited appreciable positive reactions, localized in the red pulp, probably as a result of non-specific adsorption of Pb. These differences in non-specific absorption of Pb were even more pronounced on unheated sections, the double fixed material adsorbing more Pb than the acetone-fixed sections, since normal sections generally adsorb more Pb than heat-inactivated ones.

It is somewhat difficult to account for the positive reaction obtained on incubation of formalin-acetone fixed sections in view of their slight residual DNase activity. It would undoubtedly be useful to exclude the possibility of phosphodiesterase contamination of the exogenous phosphatase used in the incubation medium [5] since this would be equivalent to having a source of DNase in the medium, thus leading to preferential staining of normal formalin-acetone fixed sections because of their greater affinity for lead as compared to normal acetone sections and heat-inactivated controls. This is not a fully satisfactory explanation since in the localization patterns obtained by Aldridge *et al.* [1] only the red pulp, but not the heavier staining ring around the lymphoid nodules, shows affinity for lead.

Acetone fixed sections, on the other hand, contain DNase II which largely diffuses during incubation so that, while the resultant staining may be due in part to enzymatic activity in the section itself, it is more probably the result of enzyme diffusion (plus possible phosphodiesterase contamination of exogenous phosphatase). Prolonged incubation times are not necessary with such spleen sections, since staining is frequently obtained after 3—5 hours incubation. It nonetheless remains to be established whether this is due, even in part, to section localized DNase.

SUMMARY

1. Histochemical localization of ribonuclease enzymes has been attempted, using nucleoside cyclic phosphates as substrates, and exogenous alkaline phosphomonoesterase in the incubation medium to dephosphorylate the nucleoside monophosphates resulting from the action of ribonuclease; the resulting inorganic phosphate is precipitated by calcium according to the Gomori procedure.

[5] The acid phosphatase used by us was prepared according to the method of Kerr & Chernigoy [32] and, on examination, was found to contain phosphodiesterase active against DNA on prolonged incubation. It should be recalled that the alkaline phosphatase used above for attempted RNase localization likewise was contaminated with phosphodiesterase, but this latter did not attack the nucleoside cyclic phosphates used as substrates during the incubation periods employed.

2. The overall reaction is proportional to the enzymatic activity; however the nuclear localization obtained, which is contrary to the results of fractionation techniques, was found to be due largely to excessive diffusion of the enzymes from fixed tissue sections.

3. Data are given with regard to diffusion from fixed tissue sections of acid and alkaline ribonucleases; and the various artifacts resulting therefrom described. Problems resulting from the use of heat and ultraviolet inactivated sections as controls are discussed.

4. Prerequisites for the histochemical localization of ribonuclease enzymes are discussed, the most important of these being the development of some method of fixation which will retain at least part of the enzyme in a tissue section.

5. The procedure of Aronson *et al.* [3] and Aldridge *et al.* [1] for the histochemical localization of deoxyribonuclease II has been re-examined in the light of the above findings for ribonucleases, with particular reference to the effect of fixation on enzymatic activity, the rate of diffusion of the enzyme from fixed tissue sections, and the validity of the resulting localization. The difficulties inherent in this method are essentially similar to those encountered with ribonuclease enzymes.

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BADANIA NAD HISTOCHEMICZNĄ METODĄ LOKALIZACJI NUKLEAZ

Streszczenie

1. Usiłowano opracować histochemiczną metodę lokalizacji enzymów typu rybonukleaz, stosując jako substrat cykliczne fosforany nukleozydów oraz dodając do środowiska inkubacyjnego fosfomonoesterazę celem defosforylacji monofosforanów nukleozydów powstałych w wyniku działania rybonukleazy. Uwolniony nieorganiczny fosforan wytrącony był przy użyciu jonów wapnia według metody Gomoriego.

2. Intensywność reakcji jest na ogół proporcjonalna do aktywności enzymatycznej preparatów. Uzyskiwana lokalizacja jądrowa, sprzeczna z wynikami uzyskiwanymi metodami frakcjonowania komórki jest w znacznej mierze wynikiem ogromnej dyfuzji enzymów z utrwalonych preparatów.

3. Przedstawiono dane dotyczące dyfuzji rybonukleazy kwaśnej i alkalicznej z utrwalonych skrawków i opisano szereg artefaktów wynikających z tego zjawiska. Omówiono również zagadnienia związane z zastosowaniem preparatów kontrolnych zinaktywowanych termicznie lub ultrafioletem.

4. Omówiono niezbędne warunki histochemicznej lokalizacji nukleaz, z których podstawowym jest opracowanie metod utrwalania umożliwiających zachowanie chociaż części enzymu w tkance.

5. Zbadano histochemiczną metodę wykrywania DNazy II według Aronson *et al* [3] i Aldridge *et al* [1] z punktu widzenia wyników uzyskanych dla rybonukleazy, ze szczególnym uwzględnieniem wpływu sposobu utrwalania na aktywność enzymatyczną, szybkości dyfuzji enzymu z preparatów parafinowych oraz specyficzności uzyskanej lokalizacji. Stwierdzono, że trudności charakterystyczne dla tej metody są zasadniczo zbliżone do trudności występujących w przypadku rybonukleazy.

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N-METHYLATION OF URIDYLIC ACID AND PREPARATION OF OLIGONUCLEOTIDES OF 3-METHYLURIDYLIC ACID

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Considerable efforts have been devoted to the synthesis of non-natural purine and pyrimidine bases as well as their nucleosides, largely in connection with attempts to isolate specific anti-metabolites. Relatively little attention has hitherto been devoted to the preparation of "non-natural" nucleotides notwithstanding that such compounds have been shown to exist in natural nucleic acids, e.g. methylated guanylic acids in ribonucleic acids from various sources [24, 22].

The preparation of 3-methyluridylic acid appeared of additional interest to us because of the eventual possibility of preparing polymers of this compound by the relatively simple chemical procedure of Michelson [17]. Such "non-natural" polymers might be expected to be of some value from the point of view of the structure of nucleotide chains; in particular a polymer of 3-methyluridylic acid should be incapable of forming a twin-stranded complex with poly-A. Synthetic "non-natural" oligonucleotides might also be expected to be useful in studies on enzyme specificity, particularly as regards polymerizing enzymes; as well as in investigations on the photochemistry of model oligonucleotides [25] currently under way in this laboratory.

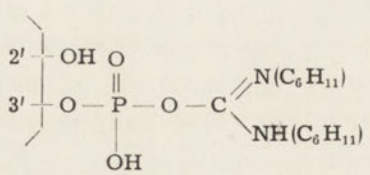
A straightforward synthesis of 3-methyluridine-2′(3′)-phosphate (V) would involve the methylation and tritylation of uridine, both of which may be achieved in high yield, followed by phosphorylation. However, phosphorylation with P_2O_5 in phosphoric acid [8, 16] of tritylated 3-methyl-uridine proved to be much too drastic in that it provoked partial demethylation and detritylation; the resulting reaction mixture contained a variety of compounds from which the isolation of III even in low yields

proved very arduous, and this procedure was abandonned after several attempts. Better results might be expected by use of a milder phosphorylating agent such as dibenzylphosphorochloridate on tritylated 3-methyluridine or by methylation with diazomethane of uridine-2′(3′)-dibenzylphosphate; we have not attempted to use this method because of the low yields normally obtained.

It was therefore decided to investigate the possibility of direct N-methylation of uridylic acid with diazomethane in anhydrous medium. Under these conditions one might expect esterification of the phosphate hydroxyl groups with the resultant formation of unstable tri-esters of phosphoric acid such as described by Brown *et al.* [3]. However in anhydrous dimethylformamide uridylic acid may be quantitatively transformed to uridine cyclic phosphate (II) [21], and subsequent methylation should then result in the esterification of only one of the phosphate hydroxyls. In view of the known lability of the cyclic phosphates in aqueous medium it might be expected that, on addition of water to the reaction medium and acidification, spontaneous opening of the cyclic phosphate ring would give a relatively stable compound, 3-methyluridine-2′(3′)-methylphosphate (IV) which could be transformed to the desired compound V by hydrolysis either in alkali [2, 3] or, as we have found, in acid. Preliminary trials encouraged us to pursue this method of attack.

We have since found that the dimethyl ester of V, resulting from the action of diazomethane on uridylic acid in anhydrous medium, is indeed rapidly hydrolyzed mainly to 3-methyluridine in 0.5 N-HCl. Under milder conditions, pH 2—3, it is transformed to the monomethyl ester in good yield, following which acidification to pH 1 results in hydrolysis to 3-methyluridylic acid. This finding is of interest in that it may prove to be a practical procedure for methylation of UMP, a possibility we are now investigating.

The procedure finally developed involved the preliminary quantitative transformation of I to II with dicyclohexylcarbodiimide (DCC) in anhydrous dimethylformamide by a modification [21] of the method of Dekker & Khorana [4]. Actually the reaction products contain, in addition to II, compounds of the form:



but these give the cyclic phosphates on slight acidification in water with the simultaneous elimination of dicyclohexylureas [4]. The addition of gaseous diazomethane to the reaction mixture in dimethylformamide resulted in the quantitative formation of the structurally interesting compound III, the appearance of which could be detected (a) spectrally, since its extinction in the ultraviolet should be the same at pH 7 and pH 12 as for 1,3-dimethyluracil [19] or 3-methyluridine; (b) chromatographically in the known solvent system propan-2-ol - NH_4OH - H_2O since it has been shown by several workers that cyclic phosphates and methyl esters exhibit appreciably higher R_F values than free nucleotides in this solvent (Fig. 1). Opening of the cyclic phosphate ring is then achieved by exposure to 0.1 N-HCl for 4 hours at 20° (or 1.5 hours at 37°). The resulting compound IV was then hydrolyzed in 0.5 N-KOH for 12—14

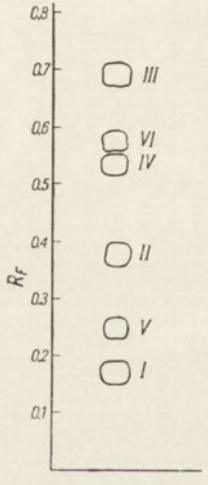
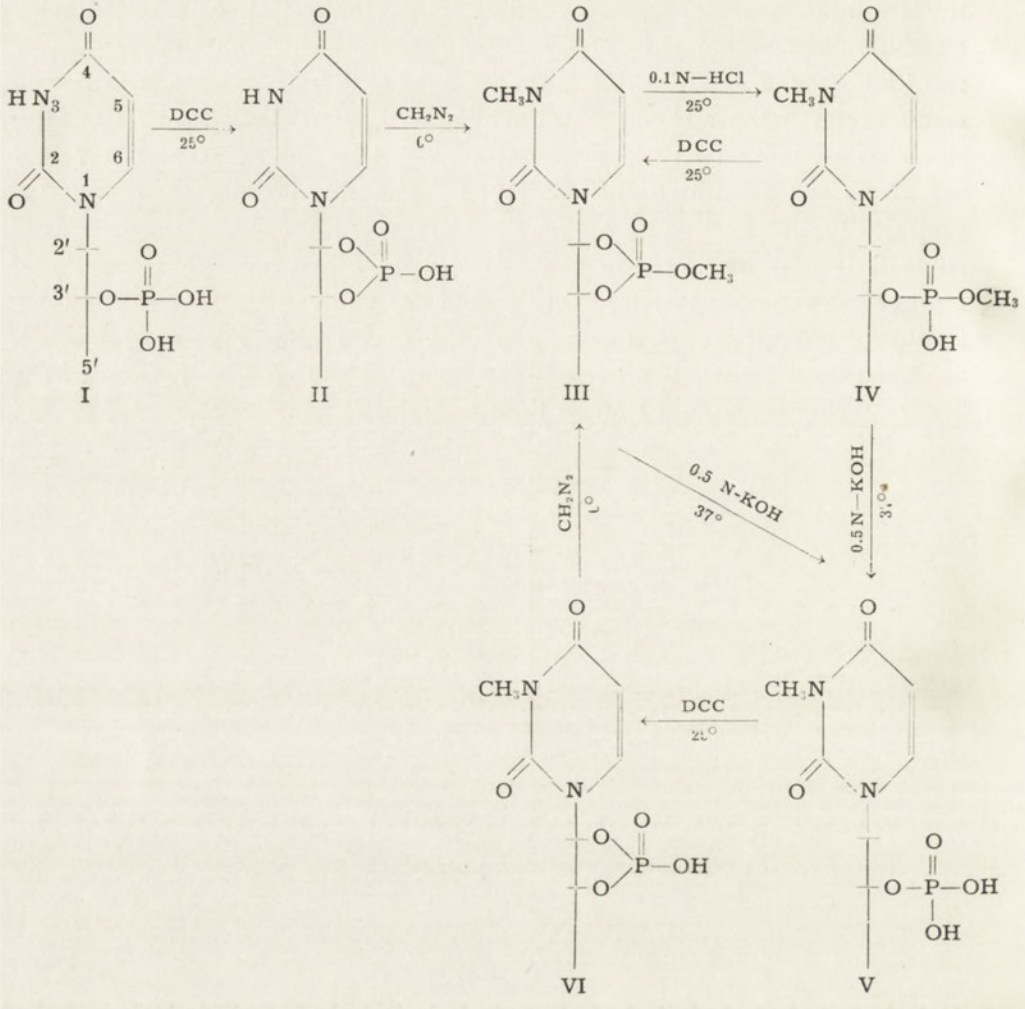


Fig. 1. Ascending chromatogram of compounds I—VI in solvent system A (propan-2-ol-ammonia-water 7:1:2, v/v/v)

hours at 37°, leading to the formation of V. It was found that V may be obtained directly from III by exposure to 0.5 N-KOH, but the incubation period required is much longer; while the use of higher temperatures or higher concentrations of alkali resulted in opening of the pyrimidine ring with the formation of a variety of decomposition products, as is to be expected from the known alkali-lability of NN′-disubstituted pyrimidones [19, 5, 20, 10].

The full reaction sequence may be illustrated as follows:



During acid hydrolysis the formation of IV is accompanied by the appearance of the methyl ester of I in about 10% yield, which is not increased on prolonged exposure to acid or alkali. The appearance of this compound is difficult to explain although it is conceivable that it is the product of demethylation of the O^4-methyl derivative formed in low yield during methylation, and which would be expected to exhibit acid lability. The removal of this compound from V was achieved on a preparative scale by taking advantage of the differential solubility of the two compounds in ethanol-ether mixtures.

Some interest attaches to the structure of compound III and there have been conflicting views as to the possibility of the existence of such

a type of "internal" triester [12]. It may be regarded as a model for the initial stage of hydrolysis of ribonucleic acid according to the well-known scheme of Brown & Todd [1], where the methyl group corresponds to the ribonucleic acid chain. The structure of this compound was confirmed by the stepwise synthesis

$$V \longrightarrow VI \longrightarrow III$$

as well as by reversal of the reaction

$$III \longrightarrow IV$$

with DCC. In aqueous medium III is, in fact, unstable and is slowly converted to IV. It is, however, more stable than the dimethyl ester of I and, in contrast to the latter [3], it may be readily chromatographed in propanol - ammonia - water in 4—6 hours without decomposition.

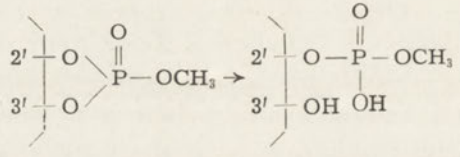
Polymers of V were obtained according to Michelson [17] with the sole exception that dimethylformamide proved to be a better solvent for V than the dioxane employed in the original method. Following isolation of the calcium salts of the oligonucleotides and opening of the terminal cyclic phosphate groups by exposure to 0.1 N-HCl for 4 hours at room temperature, chromatography in solvents A and B (see Experimental) demonstrated the absence of monomer. From the resultant mixture of oligonucleotides of varying chain lengths, di-, tri- and tetra-nucleotides may be separated on a small scale by paper chromatography (Table 1). Presumably this could be done on a larger scale by column chromatography. However, following the procedure of Michelson [17], dialysis against water was found to give a dialysate with a mean chain length of 3.3; while further dialysis of the residue against 2 M-NaCl gave a dialysate with a mean chain length of 5.5 and a residue with a mean chain length of 8.5. Chain lengths were measured by removal of terminal phosphate with prostate phosphomonoesterase prepared by the method of Loring *et al.* [13]. The relatively low percentage of long chains (Table 1) indicates that polymerization proceeds less readily than for free uridylic acid [17]. This could be due to solubility characteristics of the polymers in the solvent used for polymerization (cf. [17]) or, possibly to steric hindrance resulting from N-methylation. It would be of interest to see how readily 3-methyl-UDP would polymerize with polynucleotide phosphorylase [7] in aqueous medium where the solubility would probably be as good as for UDP itself.

The hyperchromicity of the oligonucleotide was obtained by measurement of the increase in optical density at 2620 Å on hydrolysis to mononucleotides in 1 N-HCl and is about 10% for a chain length of 3.7. This is slightly higher than the hyperchromicity of poly-U, which varies from

4.8 to 8.6% for chain lengths of 3.3 to 11.9 [17], and is suggestive of the existence of stronger interaction between the aromatic rings in the methylated polymer. It is worth noting in this connection that enzymatically synthesized, highly polymerized, polyribosethymine phosphate exhibits a hyperchromicity of up to 33% [6] as against similarly prepared poly-U which has a hyperchromicity of only 5-6% [23].

The fact that poly-methyluridine, in which the number 3 nitrogen of the pyrimidine ring is blocked, exhibits hyperchromicity (and, what is more, to an even greater extent than poly-U) provides additional confirmation for the contention that hyperchromicity in small oligonucleotides is due to interaction between π-orbitals of adjacent aromatic rings and not necessarily to hydrogen bonding [16, 17].

Particularly interesting is the behaviour of some of the above compounds towards various enzymes. For example compound IV was found to be completely resistant to ribonuclease whereas, if it existed as a mixture of 2′ and 3′ isomers, 50% should be transformed to V. The possibility was first considered that hydrolysis of III resulted in the formation only of the 2′ isomer, which would be resistant to ribonuclease:



This is, of course, contrary to what one would expect, although there is nothing in the literature on the hydrolysis of such esters. Since failure to separate the above expected isomers by column chromatography would not provide a conclusive answer to this question, we have resolved it in another way.

VI was prepared from V and was, in fact, found to be completely resistant to ribonuclease under conditions where II was completely hydrolyzed under the same conditions. Furthermore the fact that IV can be converted to III is additional evidence that it is a mixture of the two isomers and that migration of the phosphate group occurs, although perhaps less freely than in I or V, since the reaction is a slow one. It therefore follows that N-methylation of the uracil ring completely inhibits the action of ribonuclease, a fact also evident from studies of the action of the enzyme on polymers (see below). During the course of this work we came across a note [14] mentioning that Magrath & Brown had observed the inhibitory effect of N-methylation on ribonuclease activity; although these results do not appear to have been published, our own findings fully confirm them.

In agreement with the above we found that oligonucleotides of V were completely resistant to ribonuclease. Surprisingly enough, neither phosphodiesterase purified from snake venom, nor snake venom itself, exhibited marked activity against oligonucleotides of V. Our prostate phosphomonoesterase also proved to be inactive against oligonucleotides of V, thus allowing of its use for chain length determinations notwithstanding that it exhibited some activity against the calcium salt of bis (*p*- nitrophenol) phosphate. However, prolonged exposure of oligonucleotides of V to this enzyme at alkaline pH resulted in hydrolysis to 3-methyluridine, indicating the presence of some non-specific phosphodiesterase.

Alkaline phosphatase was found to rapidly dephosphorylate V, as expected. But prolonged exposure to this enzyme also resulted in the conversion of IV to 3-methyluridine, *via* V. One would consequently expect this enzyme to hydrolyze oligonucleotides of V and this is, in fact, what was found, using alkaline phosphatases from two different commercial sources.

It consequently follows that there is present, in various tissues, a phosphodiesterase which is capable of hydrolyzing both 2′, 5′ and 3′, 5′ internucleotide linkages in oligonucleotides of 3-methyluridylic acid.

EXPERIMENTAL

Attempted phosphorylation of 3-methyl-5′-trityluridine: 3-methyluridine, obtained according to Miles' modification [18] of the method of Levene [11], was tritylated in the normal way to give 3-methyl-5′-trityluridine, m. p. 170 - 171° (173 - 174° according to Levene [11]). 500 mg. 3-methyl-5′-trityluridine was added to a cooled mixture of 2 gms. P_2O_5 and 3 ml. 85% phosphoric acid, and the reaction vessel warmed slowly to 60° and left for two hours at this temperature with the exclusion of moisture. The reaction mixture was then diluted with water and brought to neutrality with saturated barium hydroxide. The reaction products were then identified by chromatography in solvents A, B and C, (see page 501) and found to include (in decreasing order of concentration) 3-methyluridine, 3-methyluridine-2′(3′)-phosphate, uridine-5′-phosphate, 3-methyluridine-5′-phosphate, and probably some diphosphates. Modification of the reaction conditions (time, temperature, ratio of nucleoside to amount of phosphorylating agent) over a wide range did not result in the formation of 3-methyluridine-2′(3′)-phosphate as the main product. In one trial a mixture of 32 mg. of the 2′(3′) and the 5′ phosphates of 3-methyluridine was extracted from the reaction mixture.

3-methyluridine-2′(3′)-phosphate (V): 500 mg. (1.6 mM) of uridine-2′(3′)--phosphate (I) was dissolved in 10 ml. dry dimethylformamide and 2.0 gms. (9.7 mM) NN′-dicyclohexylcarbodiimide (DCC) added [21]. In about 15 - 20 mins. I had completely disappeared, as shown by chromatography in solvents A and/or B. The flask containing the reaction mixture was immersed in an ice bath and saturated with gaseous diazomethane (obtained by heating under reflux an ethereal solution of CH_2N_2 from 5.15 gms. of N-methylnitrosourea and drying the gas over granulated NaOH before passing it into the reaction mixture). The course of the reaction was followed by drawing off an aliquot of the reaction mixture at 10-minute intervals and measuring the change in optical density in aqueous solution at 2620 Å on varying the pH from 7 to 13; after about 30 mins. the optical density was the same at neutral and alkaline pH, indicating that the reaction had gone to completion. Chromatography in solvents A and B now showed the absence of uridine-2′ : 3′-phosphate (II) and the appearance of only one reaction product, 3-methyluridine-2′ : 3′-methyl phosphate (III). To the reaction mixture was now added 10 ml. water and the precipitate of dicyclohexylurea filtered off. The precipitate was washed with water and the washings added to the filtrate. The latter was evaporated to dryness under reduced pressure and the residue dissolved in 40 ml. 0.1 N-HCl and left at room temperature for 8 hours. Chromatography at this point in solvents A and B showed the absence of III and the appearance of one main product, 3-methyluridine-2′(3′)-methyl phosphate (IV), along with less than 10% of the methyl ester of I (determined by elution of the two spots and measurement of their optical densities at 2620 Å). The solution was then neutralized to pH 7 with 1 N-KOH and extracted three times with 20 ml. portions of ether to remove final traces of dicyclohexylurea. The aqueous phase was then brought to about pH 13.4 by the *cautious* addition of an equal volume of 1 N-KOH and then left overnight at 37°. Chromatography in solvents A, B and C now showed the absence of IV and the appearance of a main product, 3-methyluridine--2′(3′)-phosphate (V), along with an additional minor spot corresponding to I in about 10% yield. The solution was now brought to pH 3 with 1 N-HCl and reduced in volume to about 10 ml. under reduced pressure. Water was then removed by azeotropic distillation with ethanol and benzene. The resulting KCl crystals were removed by filtration and washed with hot ethanol. The filtrate and washings were combined and evaporated to dryness under reduced pressure. The residue was dissolved in 25 ml. water and passed through a column of Amberlite IR-120 (H^+form) to remove cations. The column was washed twice with 15-ml. portions of water and the combined eluates concentrated to small volume,

followed by azeotropic distillation with benzene and ethanol to remove water. The ethanolic solution (about 8 ml.) was then filtered with a small amount of activated carbon through a G4 sintered glass filter, cooled in an ice-bath, following which ether was added dropwise in 0.5 ml. portions, with resultant formation of a precipitate. Following each addition of ether the supernatant was checked spectrally for the presence of the compound I; the latter was completely removed after addition of 3.5 ml. ether, as shown by chromatography in solvents A, B and C. The precipitate containing substance I was then removed by centrifugation and the resulting clear supernatant reduced to 5 ml., to which was added 10 ml. ether. The resulting precipitate, V, was collected by centrifugation and washed with ether. Yield of V (after drying *in vacuo*), 345 mg. (62.5% theoretical). About 50 mg. of V was dissolved in 0.5 ml. absolute ethanol and precipitated as the calcium salt by addition of an ethanolic solution of $CaCl_2$. Analysis gave P, 8.9%; N, 7.9%. Calculated for $C_{10}H_{13}O_9N_2PCa$: P, 9.2%; N, 8.3%.

$$\lambda_{max}\ 2620\ Å;\ \varepsilon_{max}\ 8.8\times10^3$$

$$\frac{\varepsilon_{2600}}{\varepsilon_{2800}} = 2.2;\quad \frac{\varepsilon_{2600}}{\varepsilon_{2500}} = 1.3;$$

Resynthesis of 3-methyluridine-2′ : 3′-methyl phosphate (III):

(a) *From 3-methyluridine-2′(3′)-phosphate (V):* 10 mg. of V was dissolved in 1 ml. dimethylformamide and 40 mg. DCC added. After 30 mins. at room temp. V had disappeared. The test-tube containing the reaction mixture was cooled to O° and saturated with diazometane as above. After about 10 mins. paper chromatography showed the presence of only one reaction product in quantitative yield with an R_F corresponding to III.

(b) *From 3-methyluridine-2′(3′)methyl phosphate (IV):* 5 mg. of IV (obtained by elution from a chromatogram on Whatman paper 3MM) was dissolved in 0.5 ml. dimethylformamide to which was then added 20 mg. DCC. After 20 hours at room temperature (the reaction goes slowly) paper chromatography in solvents A and B showed the presence of about 70% of unreacted IV and the appearance of one new reaction product with an R_F corresponding to III and in 30% yield. After 40 hours the amount of III had increased to 80%.

Behaviour in acid and alkali: Solutions of III in 1 N-HCl and in 0.5 N-KOH were placed in a thermostat at 37°. Samples for paper chromatography and spectrophotometry were withdrawn after 1 hour and subsequently at 6—8 hour intervals. After 12 hours the alkaline solution was converted quantitatively to V, with no evidence of formation of IV as an intermediary product. However, upon further exposure to alkali,

several new unidentified products (of decomposition of V) began to make their appearance.

In acid solution III is converted to V in about 4 hours, *via* IV as an intermediary product; V itself was unaffected by exposure to acid for 60 hours at 37°.

Preparation of oligonucleotides of 3-methyluridine-2′(3′)-phosphate (VII): 85 mg. (0.25 mM) of III (dried in vacuo over P_2O_5) was dissolved in 3 ml. dry dimethylformamide and 0.1 ml. (0.25 mM) tri-*n*-octylamine added. The solution was then evaporated to dryness under reduced pressure several times with the addition of dry toluene and finally dried overnigth *in vacuo* over P_2O_5. The tri-*n*-octylamine salt was then dissolved in 2 ml. dimethylformamide to which was added 80 µl. of diphenylphosphochloridate and 150 µl. tri-*n*-butylamine. After 3 hours at room temperature (by which time V had disappeared with the accompanying formation of VI), 80 µl. diphenylphosphochloridate and 150 µl. tri-*n*-butylamine were again added and the solution left overnight at room temperature, following which chromatography in solvents A and B showed that polymerization had occurred. Chromatography of a sample acidified to pH 1 for 4 hours at room temp. (to open the terminal cyclic phosphate groups) demonstrated the complete absence of V and VI. Solvent was then removed under reduced pressure, the residue dissolved in 3 ml. water and brought to pH 8.5 with 1 N-ammonia and extracted three times with 10 ml. portions of ether. Ethanol and benzene were added to the aqueous solution which was then dehydrated by azeotropic distillation under reduced pressure. The final ethanolic solution was filtered with some activated carbon through a G4 sintered glass filter and to the filtrate was added portionwise a stoichiometric amount of a 3.5% solution of ethanolic $CaCl_2$. The resulting precipitate was isolated in two steps, washed in ether and dried in vacuo over P_2O_5. The yield of the calcium salts was 24 mg. and 57 mg. (total 81 mg., about 90% theoretical).

Chain lengths of polymers: Each of the two precipitates of the calcium salt of VII was dissolved in water and dialyzed first against water for 48 hours, then against 2 M-NaCl for 48 hours. The mean chain lengths of the individual fractions were determined by measuring the terminal phosphate liberated by treatment with prostate phosphomonoesterase, with the following results (Table 1 see next page).

The smaller oligonucleotides, up to a chain length of 4, may also be separated on a small scale by paper chromatography (see Table 2).

Acid hydrolysis of VII: A sample of VII was dissolved in 1 N-HCl and left overnight at 37°. Paper chromatography in solvents A and B showed complete hydrolysis to 3-methyluridine-2′(3′)-phosphate (V).

Hyperchromicity of VII: A sample of the polymer was dissolved in 1 N-HCl and the optical density at 2620 Å immediately measured. After 14 hours at 37°, by which time hydrolysis to mononucleotides was complete, the increase in extinction at the maximum was found to be 10.3%, for a sample with a mean chain length of 3.7.

Table 1

Chain lengths of various fractions of oligonucleotides of 3-methyluridylic acid

	Dialysate against H_2O	Dialysate against 2M-NaCl	Dialysis residue
lst precipitate	61.6%	24.9%	13.5%
2nd precipitate	70.7%	22.1%	7.2%
Mean chain length (No. of residues)	3.3	5.5	8.5

Enzymatic experiments: In all experiments, identical controls not containing enzyme were used on chromatograms. Substrates were incubated at a concentration of 5 mg./ml. in the appropriate buffer with an enzyme concentration of 0.5 mg./ml. Samples were removed periodically from the incubates and controls and examined in solvents A and B.

Table 2

Paper chromatography

The following solvent systems were used in ascending chromatography Whatman paper No. 1:
A: propan-2-ol-NH_4OH (d = 0.88)-H_2O (70:10:20, v/v/v) [15]
B: ethanol - 1M-ammonium acetate (70:30, v/v) [9]
C: propan-2-ol-H_2O (70:30, v/v) [3]

Compound	R_F in solvent		
	A	B	C
Uridine	0.49	0.73	0.66
3-methyluridine	0.78	0.91	0.85
Uridine-2′ (3′)-phosphate (I)	0.17	0.25	0.35
Uridine-2′:3′-phosphate (II)	0.37	0.56	0.42
3-methyluridine-2′ (3′)-phosphate (V)	0.25	0.49	0.46
3-methyluridine-2′:3′-phosphate (VI)	0.58	0.66	0.56
3-methyluridine-2′:3′-methyl phosphate (III)	0.70	0.85	0.85
3-methyluridine-2′ (3′)-methyl phosphate (IV)	0.54	0.80	0.50
Di-(3-methyluridylic acid)	0.12	—	—
Tri-(3-methyluridylic acid)	0.06	—	—
Tetra-(3-methyluridylic acid)	0.03	0.10	—
Poly-(3-methyluridylic acid)	0	0	0

(a) *Pancreatic ribonuclease (Armour):* At pH 7.5 and 37° 3-methyluridine-2′:3′-phosphate (VI) and its polymer (VII) were completely resistant to the enzyme even after 40 hours incubation, and 3-methyluridine-2′(3′)-methyl phosphate (IV) after 140 hours incubation. Under the same conditions uridine and cytidine cyclic phosphates were completely transformed to the corresponding isomeric nucleotides in several hours.

(b) *Snake venom phosphodiesterase:* Samples were incubated in borate buffer pH 9.2, with addition of $MgCl_2$, at 37° both with snake venom as well as with purified phosphodiesterase. Oligonucleotides (VII) were found to be relatively resistant and only prolonged incubation showed slight hydrolysis under conditions where RNA and poly-U were completely hydrolyzed.

(c) *Intestinal alkaline phosphatase* (Sigma, Warthington): Incubation was at pH 8.7, borate buffer, with addition of $MgCl_2$. In 1 hour V was completely dephosphorylated to 3-methyluridine. Following 2 hours incubation of IV, chromatograms showed the presence of traces of V as well as its product of dephosphorylation, 3-methyluridine. By reducing the enzyme concentration 5-fold the amount of V was increased, thus removing any doubt as to its being an intermediate in the hydrolysis of IV to 3-methyluridine.

Incubation of oligonucleotides for 1 hour led to the appearance of dinucleoside monophosphates and traces of 3-methyluridine. Following 20 hours incubation the only product of hydrolysis was 3-methyluridine. Time for complete hydrolysis varied somewhat for enzyme preparations from different sources. If the cyclic phosphate end groups of VII are not first opened, the rate of enzymatic hydrolysis is decreased about 30%.

(d) *Prostate phosphomonoesterase:* The enzyme was prepared according to a known procedure [13] and was contaminated with phosphodiesterase active against bis (p-nitrophenyl)-phosphate and RNA. In 1 hour at pH 5.5 and 37° it dephosphorylated V to 3-methyluridine. However, IV was completely resistant even after 140 hours incubation. Incubation of VII led to the appearance in 1 hour of dinucleoside monophosphates; following several hours incubation, chromatography in solvent B showed the presence of oligonucleotides lacking only terminal phosphate groups, with R_F values of 0.04, 0.10, 0.30, 0.40, and some material at the starting point. No 3-methyluridine or V could be detected even after 20 hours incubation.

The action of this enzyme on VII was also examined at pH 9.0. Although no effect was visible after 1 to 2 hours incubation, traces of 3-methyluridine and some dinucleoside monophosphate made their appearance after 20 hours incubation.

SUMMARY

From a consideration, and trials, of various methods for N-methylation of uridylic acid, a procedure was developed *via* the following steps: (a) formation of the cyclic phosphate in anhydrous medium, (b) treatment with diazomethane in the same medium to form 3-methyluridine-2′ :3′--methyl phosphate, (c) isolation of the latter and opening of the cyclic phosphate ring in acid, (d) alkaline hydrolysis of the phosphate ester group to give the desired product. Conditions of stability and for hydrolysis of triesters and cyclic phosphate esters were established.

Oligonucleotides of 3-methyluridylic acid have been prepared according to the method of Michelson. Maximum chain lengths obtained were 8.5 and the hyperchromicity was slightly higher than for poly-U, suggesting stronger interaction between rings in the chain. Since the number 3 nitrogen in the pyrimidine rings of these oligonucleotides is blocked, this observation provides further evidence that hyperchromicity in *small* oligonucleotides is not necessarily due to hydrogen bonding, but to interaction between adjacent aromatic rings, and involving a merging of π-electron orbitals of adjacent rings.

N-methylation renders the cyclic phosphate and methyl esters of uridine completely resistant to ribonuclease. In accordance with this result, it was found that oligonucleotides of 3-methyluridylic acid are resistant to pancreatic ribonuclease; they are also relatively resistant to snake venom or purified snake venom phosphodiesterase. It was, however, found that various tissues contain an alkaline phosphodiesterase which is active against all the above compounds.

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N-METYLOWANIE KWASU URYDYLOWEGO I OTRZYMYWANIE OLIGONUKLEOTYDÓW KWASU 3-METYLOURYDYLOWEGO

Streszczenie

Rozważono i przebadano szereg możliwości otrzymania pochodnej 3-N-metylowej kwasu urydylowego. Opracowano metodę syntezy po przez kolejne etapy: (a) otrzymanie cyklicznego fosforanu 2′ : 3′ urydyny w bezwodnym środowisku, (b) działanie dwuazometanem w tym samym środowisku i otrzymanie estru metylowego 2′ : 3′-fosforanu 3-N-metylourydyny, (c) otwarcie pierścienia fosforanowego w kwasie, (d) hydroliza alkaliczna estru metylowego i otrzymanie kwasu 3-N-metylourydylowego. Ustalono warunki hydrolizy trójestrów i estru metylowego cyklicznego fosforanu.

Wykonano syntezy oligonukleotydów kwasu 3-N-metylourydylowego metodą Michelsona. Maksymalna długość łańcucha oligozwiązków wynosiła 8,5, a efekt hyperchromowy był nieco większy niż w przypadku poli-U, co świadczy o zwiększonym oddziaływaniu pomiędzy pierścieniami pirymidynowymi w łańcuchu. Ponieważ pozycja 3 azotu w pierścieniu pirymidynowym jest zablokowana grupą metylową, obserwacja ta stanowi dalsze potwierdzenie danych, że efekt hyperchromowy w małych oligonukleotydach jest spowodowany wzajemnym przenikaniem orbitali π-elektronów sąsiednich pierścieni, a nie powstawaniem wiązań wodorowych pomiędzy nimi.

N-metylacja czyni cykliczny fosforan i estry metylowe urydyny całkowicie odpornymi na działanie rybonukleazy. Zgodnie z tym oligonukleotydy kwasu 3-N-metylourydylowego okazały się również odporne na działanie rybonukleazy z trzustki; ulegają one z trudnością rozkładowi pod wpływem działania jadu węża i oczyszczonej fosfodwuesterazy z jadu węża. Jednakowoż stwierdzono, że w różnych tkankach zawarta jest alkaliczna fosfodwuesteraza, która wykazuje aktywność w stosunku do otrzymanych związków.

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PHOTOCHEMISTRY OF MODEL OLIGO- AND POLY-NUCLEOTIDES

III. CROSS-LINKING AND STAINING PROPERTIES OF ULTRAVIOLET IRRADIATED FILMS OF NUCLEIC ACIDS AND OLIGONUCLEOTIDES*

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The starting point for the present investigation was our desire to examine the staining behaviour of ultraviolet irradiated films of deoxyribonucleic acid (DNA) [1], particularly with reference to the possibility of establishing some correlation between the staining properties of irradiated DNA and cellular material. For this purpose we have used labelled dyes [4, 5] so that dye uptake and retention by a film on a glass slide may be followed by simple end-window counting [32, 33, 34] [2].

It was observed some time ago by Setlow & Doyle [26] that ultraviolet irradiation of dry films of DNA deposited by evaporation on a glass slide, under nitrogen or in a vacuum, led to the formation of a photoproduct which exhibited the consistency of a gel in aqueous or salt solutions. The efficiency of gel formation was remarkably high, the quantum yield for the process being in the neighbourhood of 10^{-2} and hence 3 to 4 orders of magnitude greater than that for the loss of viscosity of DNA when irradiated in solution [10] and at least 2 orders of magnitude greater than that for loss of biological activity by solutions of DNA (see refs. [30, 36] for reviews).

This phenomenon was subsequently further investigated by Kaplan [14], who demonstrated that the rate of gelation is linked to the state of hydration of the DNA film, the rate being increased by a factor of more than 20 as the relative humidity was decreased from 97.5% to 33%. Since

* Part II, this journal, 7, 377, 1960.

[1] The following abreviations will be used in this text: DNA, deoxyribonucleic acid; RNA, yeast ribonucleic acid; T-DNA, transforming DNA; APA, apurinic acid; poly-A, polyadenylic acid; poly-G, polyguanylic acid; poly-C, polycytidylic acid; poly-U, polyuridylic acid; poly-T, polythymidylic acid.

[2] A preliminary account of this work has already appeared [35].

a quantitatively similar dependance on humidity had previously been observed for the s-mutation and killing rates of *Serratia marcescens* [14], it was concluded that a common mechanism is involved in all three processes, which were assumed to proceed *via* hydrogen bond rupture in the DNA molecule.

In contrast to the results of Setlow & Doyle [26], we found that irradiation of a DNA film in air, with somewhat higher doses than those used by the foregoing authors, resulted in a photoproduct which, in place of the expected gel formation on contact with water or salt solution, consisted rather of relatively long fibres which were completely insoluble in aqueous solution. Furthermore the staining behaviour of these irradiated films, particulary with respect to methyl green, were likewise modified in such a way as to invite further investigation, including comparisons with variously modified forms of natural and synthetic polynucleotide chains, as well as with some microorganisms and tissue sections.

MATERIALS

DNA was obtained by a modification of the procedure of Signer & Schwander [37]; as well as from deoxyribonucleoprotein prepared according to Doty & Zubay [8] and deproteinized first by the procedure of Marco & Butler [18] and finally according to Sevag *et al.* [28]. Both preparations were highly polymerized. We are indebted to Dr. F. Koerner for a generous gift of glucosylated DNA from phage T_{2r} and to Dr. R. Pakula for a sample of pneumococcal transforming DNA.

Yeast RNA was either a commercial preparation of the sodium salt or a more highly polymerized sample prepared according to the method of Chantrenne [7] as the free acid and dissolved by the cautious addition of dilute NaOH to neutrality.

APA was prepared from thymus and phage DNA's according to the methods of Tamm *et al.* [39] and Durand & Thomas [9]. Reduced APA was prepared by sodium borohydride treatment according to the procedure of Hurlen *et al.* [13]. and deaminated APA by application of the method of Vandendriesche [41] for deamination of RNA.

Poly-A [12] was a gift of Dr. S. Ochoa. Poly-U and poly-G were chemically synthesized preparations [19, 20] for which we are indebted to Dr. A. Michelson; both of these consist of chains containing a mixture of 2′, 5′ and 3′, 5′ internucleotide linkages and with chain lengths of about 15 residues. A sample of poly-C [12] was kindly provided by Dr. M. Manago.

Poly-T was isolated from DNA following thermal degradation of the previously prepared APA and column chromatography of the degradation

products [1]. The sample of poly-T used was that eluted at high salt concentrations. It was dialyzed exhaustively against 2 M-NaCl and then against water, so that the chain length is probably at least 10 residues, perhaps greater. More than 90% of this oligonucleotide was hydrolyzed by snake venom to thymidine.

METHODS

All substances were deposited from aqueous solution on glass slides and air dried, usually at about 60° in an oven. Drying at this temperature was found to be more convenient than slow air-drying at room temperature and was used in most experiments after it had been demonstrated that the final results were the same. The resulting dried films were about 10 mm. in diameter and contained usually about 25 μg of material. The absorption of such a film at 2537 Å was about 70%.

The slides were then exposed to the radiation (2537 Å) from a Phillips 30-watt germicidal lamp, usually at a distance of 5 cms. from the lamp surface, the incident intensity being then about 100 ergs/mm².sec., as measured by uranyl oxalate actinometry.

Irradiation was conducted with the films exposed to atmospheric conditions. Several experiments were carried out with films deposited on the inner surface of closed quartz cuvettes through which dried nitrogen had been circulated; since the results obtained were qualitatively similar, this procedure was not adhered to.

Unless otherwise stated the films were stained on the glass slides with 0.25% dye solutions in 0.1 M-acetate buffer pH 4.1 [15]. The preparations were covered with about 20 μl. dye solution and placed in covered Petri dishes saturated with water vapour to prevent evaporation. After 15 minutes, the preparations were removed, differentiated with 95% ethanol for 15 seconds and air-dried. The amount of dye retained by the preparation was then obtained by end-window counting, using a 2 mg. window counter (see next paragraph).

The preparation of ^{35}S-labelled thiazine [4] and ^{14}C-labelled triphenylmethane [5] dyes has been previously described, as have also the procedures used in staining with such dyes on glass slides [29]. The technique of Gram staining with labelled iodine has likewise been reported elsewhere [32, 33].

RESULTS AND DISCUSSION

The effect of irradiation on the retention of methyl green by a film of DNA is illustrated in Fig. 1, from which it will be observed that the behaviour of DNA from sources as widely different as calf thymus,

bacteria and a bacteriophage, is qualitatively similar. The affinity of the films for methyl green increases with time of irradiation up to about 1 1/2 — 2 hours, at which time it is from 50 to 90% higher than that of the non-irradiated controls. With prolonged irradiation the staining affinity subsequently slowly decreases.

If, instead of staining an irradiated film, the glass slide on which it is deposited is immersed in distilled water, the DNA does not dissolve but passes into the aqueous phase as fibres which are initially extremely fine. Fibre formation is readily visible under the microscope following several

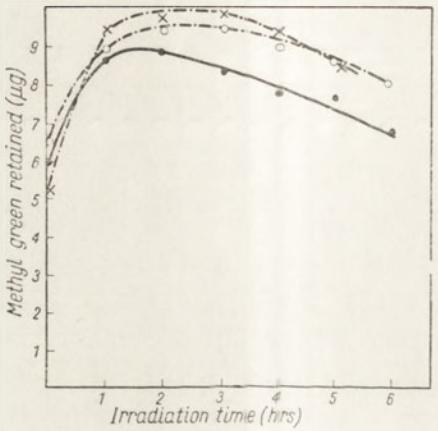


Fig. 1. Effect of ultraviolet irradiation on retention of methyl green by films of DNA from various sources; (x) *D. pneumococci*, (o) calf thymus, (o) bacteriophage T2

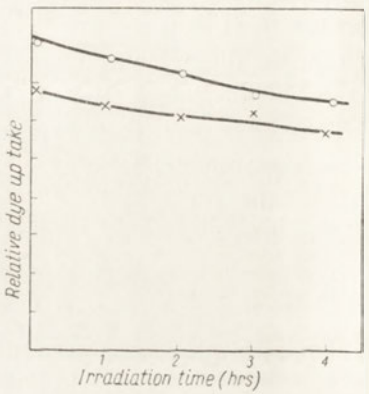


Fig. 2. Effect of ultraviolet irradiation on retention of methylene blue (○) and crystal violet (x) by films of calf thymus

seconds irradiation; this is best done by depositing a drop of water on the irradiated film and observing under low magnification. The dimensions of these fibres, or threads, increases with time of irradiation so that, after several minutes, they may be seen with the naked eye; following about 1—2 hours irradiation they attain a length of up to 7—8 mm. and a diameter of about 200 μ. With further exposure to the irradiation source, the fibres are degraded, albeit very slowly.

It should be noted that there is a relatively close parallelism between fibre formation and affinity of the irradiated films for methyl green.

If the irradiated films are exposed to dilute saline or alcohol in place of water, somewhat smaller fibres are formed. Furthermore the shape of the fibres formed in water varied according to the type of DNA used, bacteriophage DNA, e.g. forming threads which were somewhat spiral in

form. Thymus DNA was, however, generally used in subsequent experiments.

Particularly striking is the fact that the fibres are completely insoluble in water or concentrated salt solution, even at elevated temperatures. Furthermore, exposure of a suspension of fibres in aqueous medium at pH 7.5 to the prolonged action of deoxyribonuclease is completely without effect.

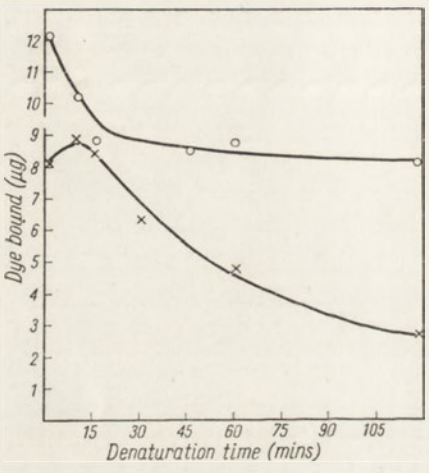
Some idea as to the rate of formation of photoproduct may be obtained by following the rate at which the DNA is rendered insoluble during the very early stages of the reaction. This was done by irradiating larger quantities of material (about 100 μg.) which was taken up in 3 ml. water, centrifuged, and the optical density of the supernant read at 2600 Å. It was found in this way that more than 70% of the DNA was rendered insoluble following 1 minute irradiation. Following several minutes irradiation no DNA could be detected in solution. These results differ in some respects from those of Setlow & Doyle [26], who also studied this early phase of the reaction. The rate of insolubilization of DNA is several-fold slower than that found by the foregoing authors; this could be due to the fact that their films were vacuum dried and then irradiated either in vacuum or under dry nitrogen, while ours were air-dried and exposed to atmospheric conditions during irradiation so that they must have been hydrated to some extent (cf. ref. [14]). It should, however, be noted that the DNA preparations used by the foregoing authors were such that not all the DNA could be rendered insoluble with higher radiation doses. A more serious contradiction is the fact that our DNA was rendered insoluble in 0.1 M-NaCl at a slower rate than in water; whereas the above authors report their irradiated DNA to become insoluble at a more rapid rate in salt solution.

If DNA films, prior to and after irradiation, are stained at alkaline pH, the overall results are similar. It is, however, more convenient to stain at acid pH because of the known alkaline instability of methyl green.

Since it might be argued that the increase in dye uptake of irradiated films is due, in whole or in part, to trapping of dye molecules in the fibre interstices, several comparative experiments were carried out in which staining was done with dyes other than methyl green. If the staining is done at alkaline pH, irradiated films show no change in affinity for thionine, a 15% increase for methylene blue, and no change for acid fuchsin. With the normal staining procedure at pH 4.1 there is a decrease in staining affinity with time of irradiation for methylene blue, as well as for crystal violet (Fig. 2). It follows, therefore, that the methyl green staining of irradiated DNA films involves some degree of specificity.

If DNA is heat denatured or degraded with desoxyribonuclease the resultant affinity for methyl green is considerably reduced. However, irradiation of films of the products of thermal denaturation or enzymatic degradation leads to a marked increase in affinity for methyl green (Figs. 3 and 4), as well as fibre formation on exposure to water.

From Fig. 3 it will be observed that non-irradiated heat denatured DNA shows initially a small increase in dye affinity. This phenomenon is quite reproducible, although not readily explicable since heat denaturation is supposed to destroy the double stranded structure presumed to be necessary for specific binding of methyl green by DNA [24]. It is con-



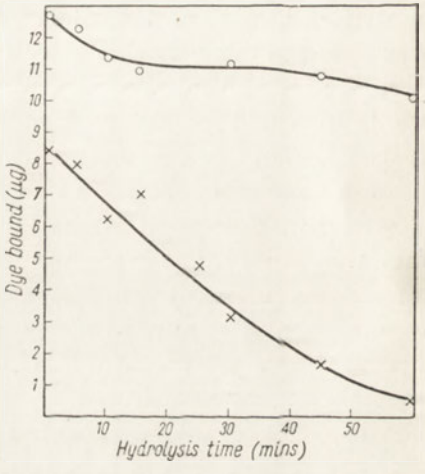


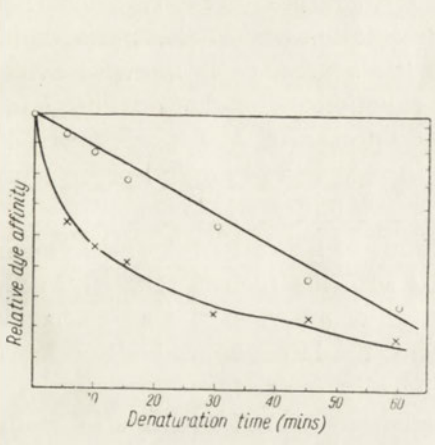
Fig. 3. Influence of prior heat denaturation at neutral pH on retention of methyl green by DNA film. Solution of DNA, 1 mg./ml., heated at 100° C; at given time intervals two aliquots of 25 µl each were withdrawn and evaporated on glass slides. One of these was then irradiated for 1 hour, following which both slides were stained: (x) heat denatured, non-irradiated, (o) heat denatured, then irradiated for 1 hour

Fig. 4. Retention of methyl green by film of enzymatically hydrolyzed DNA before (x) and after (o) irradiation for 1 hour

ceivable that slight aggregation occurs during the initial stages of heat denaturation of DNA, analogous to that encountered in the heat denaturation of some proteins. Rice & Doty [22] have, in fact, observed such aggregation in solutions of thermally denatured DNA at higher concentrations. If, however, the films are stained according to one of the procedures used for staining of tissue sections [16], and involving a rinsing in 0.1 N-HCl and washing for 5 minutes in acetate buffer prior to staining, and differentiation in acetate buffer following staining, there results

a smooth decrease in staining affinity with degree of denaturation (Fig. 5) which is, in fact, an exponential curve [3].

With a view to gaining more information about the nature of the photopolymerization process, attention was then directed to simpler, polynucleotide chains. Initial trials involved irradiation of APA as well as reduced and deaminated APA and RNA. A typical set of results is



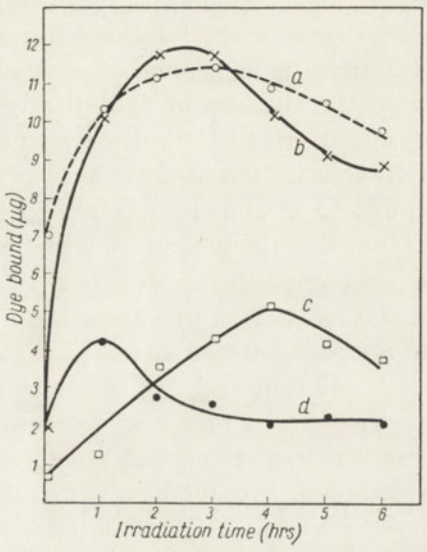


Fig. 5. Decrease in affinity for methyl green of films of thermally denatured DNA, rinsed in 0.1 N-HCl and washed for 5 mins. in acetate buffer pH 4.1 prior to staining (x). Same observations an a semi-log plot (o)

Fig. 6. Effect of ultraviolet irradiation on affinity for methyl green of irradiated films of DNA (o), APA (x), reduced APA (□) and RNA (●)

presented in Fig. 6. Undoubtedly the most striking of these is the curve for APA, a substance which normally exhibits minimal affinity for methyl green. It will be observed from Fig. 6 that, following only a few minutes irradiation, the dye affinity of APA is equal to, or even greater than, that of DNA. Irradiated APA also forms fibres in aqueous medium with dimensions comparable to those resulting from irradiated DNA, but differing from the latter in that they exhibit a greater uniformity in structure [4].

[3] It is perhaps worth pointing out that the above procedures, using labelled dyes, are quite convenient technically for following the kinetics of enzymatic hydrolysis or thermal denaturation of DNA.

[4] APA prepared according to the procedure of Durand & Thomas [9] gave shorter fibres than that prepared according to Tamm et al. [39]. This is most likely related to the differences in structure between these two different preparations.

Representative photographs of irradiated DNA and APA fibres, as observed under low magnification, are shown in Figs. 7a, 7b, 7c, and 7d.

Since APA has a molecular weight of about 15,000 [40] and consequently a chain length about 1% that of the parent DNA from which it was derived, it follows from the above results that the molecular weight of the polynucleotide chain is not of primary importance in determining the extent of photopolymerization. It is therefore of interest that RNA forms small and ill-defined fibres on irradiation and is also more rapidly degraded upon prolonged exposure to the source, as is likewise evident from the changes in dye affinity as a function of irradiation time (curve *d*, Fig. 6). Reduction of the free aldehyde groups of APA prior to irradiation also results in a marked decrease in response to methyl green (curve *c*, Fig. 6) as compared to normal APA (curve *b*, Fig. 6).

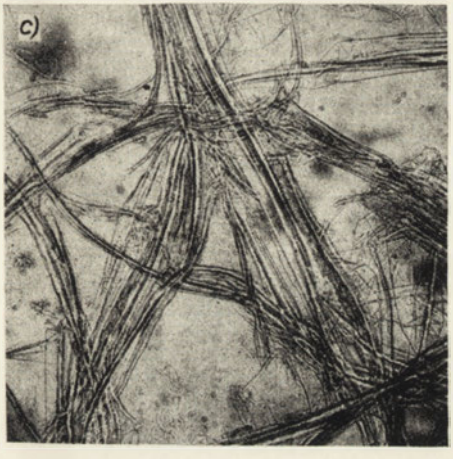
On the other hand the aldehyde groups of normal APA are, following irradiation, still free and readily stained with the fuchsin reagent. In fact, if the fibres resulting from the irradiation of an APA film are suspended in a dilute solution of the fuchsin reagent, the latter is gradually removed from solution and the fibres become heavily stained.

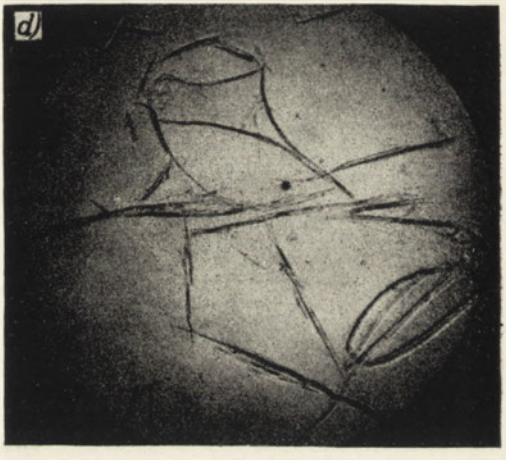
The fibres resulting from the irradiation of DNA will also readily remove the dye from a solution of methyl green in which they are suspended. Presumably this could also be used as a quantitative method for staining DNA threads, by optical measurement of the dye remaining in solution, but we have not investigated this further.

It may reasonably be concluded from the preceding experiments that the process of aggregation, or cross-linking, is not a random phenomenon but involves some degree of specificity with respect both to base composition of the nucleotide chain as well as, to some extent, of its structure. A series of trials was therefore initiated with some model synthetic polynucleotides.

It was first established that enzymatically prepared poly-A, following irradiation, exhibited no modification in affinity for dye, nor was fibre formation observed upon exposure to water or salt solution. It is of interest, in this connection, that twin-stranded poly-A (formed by acidification to pH 5.2 of a solution of poly-A [11]) exhibits a marked affinity for methyl green. It is perhaps also pertinent at this point to draw attention to the high radiation resistance of purines and purine oligonucleotides as compared to pyrimidines [43].

The results for poly-G were likewise negative. Some exception could be taken to this finding on the grounds that poly-G differs from a natural oligonucleotide chain in that the internucleotide linkages are a mixture of 2′, 5′ and 3′, 5′, this being a consequence of the chemical procedure





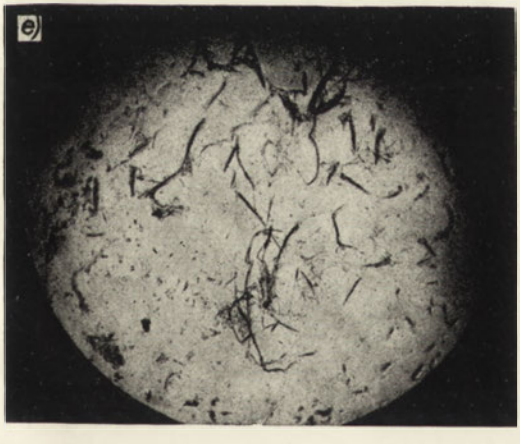


Fig. 7. Appearance under the microscope of: a) DNA irradiated 1 hour, then placed in water (110×); b) DNA film irradiated 1 hour, then exposed to water and dried (110×); c) APA film irradiated 1 hour and placed in water (110×); d) APA film irradiated 15 mins., placed in water and then dried (75×); e) Poly-U film irradiated 1 hour, placed in water and then dried (75×)

used for the synthesis of ribooligonucleotides [19, 20]. While it would obviously have been more satisfactory to use a poly-G with only 3′, 5′, internucleotide bonds, this is at the moment out of the question because of the inability of polynucleotide phosphorylase to synthesize homopolymers of guanylic acid.

The validity of the above observations for poly-G is, on the other hand, indirectly vouched for by the behaviour of chemically synthesized poly-U, which also includes a mixture of 2′, 5′ and 3′, 5′ internucleotide linkages. Irradiated films of poly-U form threads of appreciable size in aqueous medium (Fig. 7e), while the uptake of methyl green with time of irradiation is very marked (Fig. 8), as for APA (Fig. 6, curve *b*). It is

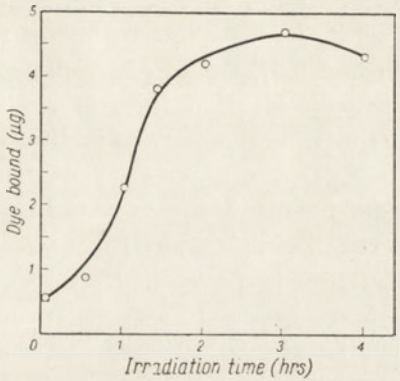


Fig. 8. Uptake of methyl green by film of irradiated poly-U

also not without interest that the average chain-length of the poly-U used was about 10—15, so that the molecular weight is only about one-fifth to one-quarter that of APA.

Enzymatically synthesized poly-C was also found to exhibit radiation induced aggregation, but only partially in the form of threads. Dye uptake, on the hand, increased considerably with time of irradiation.

Some trials were carried out on films of polysaccharides and of some proteins. The former were completely inert, as might be expected from their lack of absorption in the ultraviolet; no attempts were made to irradiate at wavelenghts below 2200 Å where carbohydrates begin to exhibit end absorption. Some proteins were found to exhibit an increased affinity for dyes upon irradiation, but without formation of water insoluble fibres; this does not, however, exclude the possibility of formation of water soluble aggregates, as occurs during, irradiation of aqueous solutions of some proteins [23].

Setlow & Doyle [26] have reported that deuteron and electron irradiated DNA films form gels in salt, but not aqueous, solution. We have subjected DNA and APA films to the radiation from a ^{60}Co source. With doses varying from 10^5 to 2×10^6 rads no evidence for fibre formation or increase in dye uptake could be observed. A few trials were carried out with 0.5 MeV deuterons with positive results, although not as clearly defined as with ultraviolet irradiation. This is in accord with the earlier observations of Setlow & Doyle [26] and gains added significance as a result of the recent report of Alexander & Stacey [2] on the *in vivo* polymerization of DNA by electron irradiation.

It has previously been shown that films of RNA and APA take the Gram positive stain, whereas DNA films are gram-negative [31]. By contrast an ultraviolet irradiated film of DNA is strongly Gram positive (Fig. 9). Furthermore, while Gram stained films of RNA and APA decolorize very rapidly in alcohol [33], a film of irradiated DNA takes the stain so effectively that its decolorization time is similar to that for many strains of gram-positive microorganisms. In addition the iodine in a gram-stained film of irradiated DNA can undergo exchange in the same way as gram-stained microorganisms [32, 33] indicating that the iodine is bound in the ionic form. Whereas the half-time for exchange of the iodine in gram-stained Saccharomyces is 12 secs., that for irradiated DNA is 80 secs, as compared to 55 secs. for acid-fast organisms. It should be noted from Fig. 9 that affinity for the Gram stain increases much more rapidly with time of irradiation than does affinity for methyl green and is also lost more rapidly with prolonged irradiation. It is also clear from Fig. 2 that modification of the Gram staining behaviour as a result of irradiation is not due simply to a change in affinity for crystal violet.

In view of the foregoing results, it is pertinent to inquire to what extent, if any, such aggregation or cross-linking processes may occur intracellularly as a result of ultraviolet irradiation. An unequivocal answer to this question would, of course, require extraction of the cellular constituents following irradiation. However, the poor solubility characteristics of irradiated polynucleotides at neutral pH in aqueous or salt solutions would not be expected to facilitate such a task. By a judicious choice of material, viz. fish sperm heads which consist essentially of DNA and protamine, Alexander & Stacey [2] succeeded in demonstrating, by an extraction procedure following exposure to ionizing radiations, that cross-linking of DNA does occur when the latter is linked to protein *in vivo*.

We have attempted, by means of staining techniques, to obtain some indication as to whether ultraviolet irradiation of cellular material is

accompanied by cross-linking. While the results cannot be regarded as conclusive, they do suggest that such processes are operative at least to some extent.

The uptake of methyl green by unfixed smears of gram-positive organisms such as *Bac. subtilis* and *Saccharomyces* (fixed after irradiation) decreases with time of irradiation. On the other hand a gram-negative organism such as. *E coli* exhibits an increase of almost 100% in methyl green uptake during 1—3 hours irradiation, followed by a relatively slow decrease with prolonged irradiation.

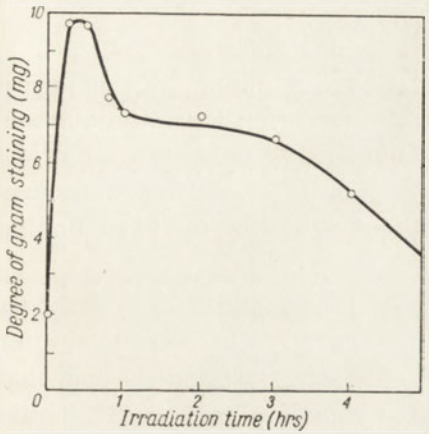


Fig. 9. Variation of degree of gram-positivity of film of irradiated DNA as function of time of irradiation

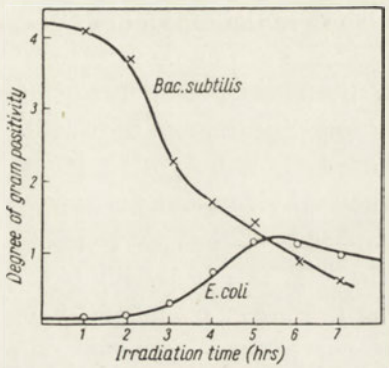


Fig. 10. Variation of degree of gram-positivity of smears of *Bac. subtilis* and *E. coli* as a function of time of irradiation. The smears were heat-fixed *after* irradiation, prior to staining

The gram-positivity of gram-positive organisms was found to decrease as a result of irradiation, as demonstrated many years ago by Cernovodeanu & Henri [6] and subsequently confirmed by Bartholomew & Mittwer [3]. The use of labelled iodine makes it possible to follow this process quantitatively [29, 32, 33] as can be seen from Fig. 10. Particularly interesting, however, is the behaviour of a gram-negative organism such as *E. coli;* from Fig. 10 it will be seen that, following a lag period of about 2 hours, the cells become pronouncedly gram-positive. The stain is not taken up uniformly but is localized in various "granules", indicating involvement of some specific cell constituents. Spores were also found to become gram-positive on irradiation, to an even greater extent than *coli.*

Irradiation of rat liver tissue sections was found to result in increased methyl green uptake in the nuclei. Upon prolonged irradiation, followed

by staining with methyl green-pyronine, it was found that increased dye uptake was not confined to the nuclei alone. An examination was also made of the staining behaviour towards methyl green of Feulgen--hydrolyzed sections, in which the nuclear DNA has been replaced by APA, with accompanying loss of affinity for methyl green. Although the results were not always reproducible, it was found in several experiments that irradiation of such sections restored to a small extent the affinity of the nuclei for methyl green, as would be expected from APA aggregation under the influence of ultraviolet light.

CONCLUDING REMARKS

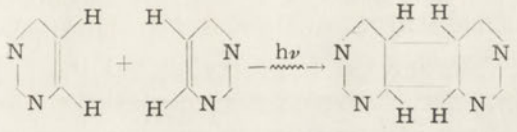
The radiation-induced aggregation or cross-linking of biological polymers such as nucleic acids by ultraviolet radiation is not unique and has been observed for such synthetic polymers as poly (vinylchloride) [38] and linear polyesters [42]. In addition photosensitized reactions may be used to induce with a fairly high degree of efficiency the cross-linking of polyethylene and polyacrylamide [21].

In the absence of concrete data as the nature of the new linkages formed, it is not possible to calculate directly the quantum yields for cross-linking of nucleic acid films. However, bearing in mind the fact that fibres are visible after only a few seconds irradiation with the moderate intensities employed, the efficiency of the process must be very high indeed. Setlow & Doyle [26] calculate a quantum yield for their findings of about 10^{-2}, based on the loss of solubility of the DNA film and corresponding therefore to the initial stages of the reaction. If we employ the same criterion for our results, the corresponding value is about 3×10^{-2}, a reasonably good agreement if we recall the differences in irradiation conditions and in DNA samples (see above). These figures are 2—3 orders of magnitude greater than quantum yields for loss of biological activity or viscosity of DNA irradiated in solution, and suggests that a different mechanism is involved. Additional evidence for this is forthcoming from the observation that the rate of cross-linking is unaltered at low temperatures [26], a finding which has been proposed as a tool in action spectra investigations, since quantum yields for proteins at low temperatures are appreciably modified [25]. However, Lerman & Tolmach [17] have now shown that the quantum yield for loss of activity of T-DNA in solution undergoes a marked drop at low temperatures. As a result of this the finding that the quantum yield for the cross-linking reaction is wavelength independent [26] may likewise be inapplicable to nucleic

acids in solution, a matter of considerable importance in action spectra studies [25, 27, 30, 36].

The proposal of Kaplan [14] that the cross-linking reaction is due to hydrogen bond rupture is perhaps an oversimplification. It is indeed difficult to visualize a reaction of this nature involving native DNA molecules without rupture of at least some of the interchain hydrogen bonds. On the other hand, heat-denatured DNA, in which most of these bonds have probably been destroyed, undergoes aggregation fairly efficiently. Furthermore, although it is questionable that APA or low molecular weight poly-U are hydrogen bonded to even a small degree, both of them readily undergo cross-linking. Of greater significance is the apparent importance of pyrimidine rings in the reaction, as well as the increased specificity of the cross-linked photoproducts for methyl green. If we accept the evidence that the methyl green specificity of DNA is due to its two-stranded Watson-Crick structure [24], then the foregoing facts imply the formation of a more ordered structure as a result of irradiation.

Bearing in mind the apparent specificity of the pyrimidine rings in the reaction, one conceivable mechanism could be that involving the formation of new linkages across the 5,6 double bonds, as follows, with



resultant saturation of the latter. A somewhat analogous mechanism has been postulated for the ultraviolet-induced cross-linking of linear polyesters [42]. Since the above type of reaction involves saturation of the 5,6 double bonds of the pyrymidine rings, it should be accompanied by a loss of the characteristic absorption maximum at 2600 Å. A film of poly-U was therefore deposited on a quartz plate and its absorption spectrum examined as a function of time of irradiation; the characteristic maximum at 2600 Å was, in fact, found to decrease with time of irradiation. It would obviously be desirable to follow up this result and to confirm that it is obtained when the film is irradiated in the absence of atmospheric moisture, since predominant reaction of poly-U in solution is the uptake of a water molecule at the 5,6 double bonds with an analogous decrease in absorption of the principal maximum [33, 43]). However, the fact the rate of the cross-linking reaction for DNA films is *increased* with *decrease* in atmospheric humidity [14] suggests that it is only the cross-linking reaction which takes place. We have obtained some evidence that such a reaction may also proceed in solution, but with

a considerably lower efficiency. More direct evidence for the above mechanism obviously requires further analysis of the photoproduct.

On the whole, it is quite conceivable that reactions of the above type may occur, at least in part, in ultraviolet irradiated living cells. In fact, insofar as intracellular DNA is concerned, it is probably better represented *in vitro* by a film exposed to atmospheric humidity rather than by a solution. There are, indeed, a number of observations pointing to a higher radiation sensitivity of intracellular DNA as compared to isolated DNA in solution, although the increased sensitivity *in vivo* is not always as high as might be expected from the behaviour of irradiated films [30, 33, 36].

It should also be noted that irradiated films of bacteriophages will not subsequently undergo photoreactivation, a phenomenon which has hitherto been rather puzzling, but which is quite understandable in the light of the above mechanism. It is rather unlikely that the above type of cross-linking reaction would be susceptible of reversibility. It is consequently also to be expected that irradiated films of transforming DNA will also show negative results as regards photoreactivation; no such experiments have, however, hitherto been reported.

Acknowledgments: We should like to thank the Microbiological Committee of the Academy of Sciences for financial support to one of us (J. B.), to K. L. Wierzchowski for carrying out the experiment on the effect of irradiation on the absorption spectrum of a film of poly-U, and to Miss Z. Tramer for assistance with the deuteron and γ-irradiations.

SUMMARY

1. Ultraviolet irradiation (2537 Å) of air-dried films of deoxyribonucleic acid leads to the formation of fibres insoluble in water or salt solution and resistant to the action of deoxyribonuclease. The efficiency, or quantum yield, of the process is relatively high.

2. Irradiated DNA films exhibit an increased affinity for methyl green; this is the most convenient procedure for following the reaction, using labelled dye.

3. Experiments with model oligo- and polynucleotides show that it is mainly the pyrimidine residues that are involved in this cross-linking reaction provoked by irradiation. A tentative mechanism for the cross-linking process is proposed involving cross-linkages between the 5,6 double bonds of pyrimidine rings.

4. Irradiated DNA films are also strongly gram-positive and as resistant to decolorization as some gram-positive microorganisms.

5. Attempts have been made, by means of staining reactions, to demonstrate whether the cross-linking reaction may occur in ultraviolet irradiated cellular material. The results are not conclusive, but there is some evidence that this may be the case.

6. There was no evidence of cross-linking under the influence of γ-irradiation, but positive results were obtained with deuteron irradiation.

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POLIMERYZACJA I WŁAŚCIWOŚĆ BARWIENIA SIĘ BŁON KWASÓW NUKLEINOWYCH I OLIGONUKLEOTYDÓW NAŚWIETLANYCH PROMIENIAMI ULTRAFIOLETOWYMI

Streszczenie

1. Błony kwasu dezoksyrybonukleinowego wysuszone w powietrzu, anastępnie naświetlone promieniami ultrafioletowymi (2537 Å) tworzą włókna nierozpuszczalne w wodzie oraz w roztworach soli nieorganicznych i odporne na działanie dezoksyrybonukleazy. Wydajność kwantowa procesu jest dość wysoka.

2. Naświetlone błony DNA wykazują zwiększone powinowactwo do zieleni metylowej; zastosowanie barwników znakowanych jest najdogodniejszą metodą badania tych reakcji.

3. Doświadczenia z modelowymi oligo- i polinukleotydami wykazują, że w reakcjach polimeryzacji wywołanych naświetleniem ultrafioletowym biorą udział głównie zasady pirymidynowe. Przedstawiono hipotezę mechanizmu procesu polimeryzacji.

4. Naświetlone błony DNA są silnie gram dodatnie, a ich odporność na odbarwianie odpowiada odporności niektórych gram-dodatnich mikroorganizmów.

5. Przez zastosowanie reakcji barwnych usiłowano stwierdzić czy reakcja polimeryzacji występuje również w materiale komórkowym naświetlonym promieniami ultrafioletowymi. Nie uzyskano definitywnych wyników, ale istnieją pewne dane przemawiające za taką możliwością.

6. Nie stwierdzono zjawiska polimeryzacji pod wpływem promieni γ, natomiast napromieniowanie deuteronami dało wynik dodatni.

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STUDIES ON LIVER AUTOPSY SPECIMENS IN GLYCOGENOSIS. DISCREPANCIES BETWEEN *IN VITRO* EXPERIMENTS AND *IN VIVO* OBSERVATIONS*

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This paper is dedicated to Professor Włodzimierz Mozołowski in honor of his 65th birthday.

Liver Glycogen Disease was first recognized as a new disease entity in childhood by Wagner and Parnas [9] in 1921. The low fasting blood sugar and its failure to rise following injection of adrenalin were interpreted to be the result of a defect in mobilizable glycogen. In 1929 von Gierke [2] reported the autopsy findings of a typical case, and Schönheimer [6] pointed out the possible underlying enzymatic defect. Not until the fundamental investigations of Cori, Cori, and coworkers [1] was the enzymatic defect and the glycogen structure in the disease fully understood and a classification in different subgroups attempted. The most common form is the hepato-nephromegalic type where a deficiency of glucose-6-phosphatase, essential for the maintenance of normal blood sugar levels, is considered the clue to the pathogenesis. Structure of the liver glycogen in these cases is normal.

Recently Lowe *et al.* [7] have pointed out that detailed and critical examination of seven patients with liver glycogen disease makes suspect the accepted theories of the enzymatic pathogenesis. Striking discrepancies have been observed by these investigators between clinical biochemical findings, results of liver slice incubation, and hepatic enzyme measurements.

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The detailed report will be published elsewhere.

In one subject, for instance, with 10 per cent liver glycogen, low muscle glycogen, and normal hepatic phosphorylase and glucose-6--phosphatase, severe and sustained fasting hypoglycemia was observed. Glucagon induced glycogenolysis both *in vivo* and during incubation of liver slice incubation. In another subject with 10 per cent liver glycogen In another subject with 9 per cent liver glycogen and normal muscle glycogen, glucose-6-phosphatase was absent and phosphorylase markedly diminished, but the patient had less hypoglycemia and responded to glucagon by elevation of blood sugar and by production of glucose during liver slice incubation. In another subject with 10 per cent liver glycogen and normal muscle glycogen phosphorylase was normal, but glucose-6--phosphatase absent. The patient had only mild hypoglycemia and produced under the influence of glucagon some glucose *in vivo,* though very little *in vitro.* Five patients showed strikingly high levels of blood lactate during fasting hypoglycemia. The hyperlactecemia was reduced by feeding or glucose infusion and augmented by glucagon administration either in the fasting state or postprandially. An acute decrease in liver size was observed in four of these children following intravenous infusion of high doses of glucagon. The authors [7] conclude from their studies, that glucagon induced glycogenolysis, that lactate rather than glucose was the principal *in vivo* endproduct of glycogenolysis, and that glycogenolysis occurred spontaneously in response to hypoglycemia. In contrast, two patients, one of whom lacked hepatic glucose-6-phosphatase, had normal fasting blood lactate levels and responded to glucagon by a fall of lactate to abnormally low levels. In one of these children an abnormal rise of the blood lactate level was observed as an effect of feeding.

Our own studies during the last years have likewise yielded some results which are not easily explainable by the current theories. From autopsy material, collected from two patients with glycogen storage disease, the following experimental results were obtained: In a reaction mixture containing liver homogenate dialyzed against distilled water for 4 hours, and equivalent to 333 mg. fresh liver per ml. reaction mixture, phosphate buffer of pH 7.2, 0.001 M-adenylic acid, and 0.01 M-magnesium sulfate final concentration, considerable amounts of a fermentable sugar chromatographically identified as glucose, were formed during incubation for one hour. The livers appeared to be capable of forming *in vitro* sufficient amounts of glucose to keep the blood sugar at normal levels. This phenomenon cannot be explained by the activity of amylo-1, 6-glucosidase. A portion of the glycogen prepared from one of the livers was incubated with a pure phosphorylase; the limit dextrin resulting

from this procedure was further incubated with an amylo-1,6-glucosidase prepared from rabbit muscle. The amount of glucose found in the reaction mixture after incubation was only one sixth of that resulting from incubation of a liver homogenate which contained the same amount of glycogen. Diastatic activity as a source of this sugar could be ruled out; the formation of fermentable sugar in the presence of phosphate buffer exceeded that in specimens without inorganic phosphorus by over 300 per cent.

Not only the specific liver glucose-6-phosphatase activity was zero at pH 6.8, but also the level of alkaline phosphatase tested against β-glycerophosphate as substrate was considerably low (cf. Thannhauser *et al.* [8]. Phosphorylase activity, measured according to Hers [3] amounted in one of the two livers to 25.3 μmol per g. liver per minute which is within normal limits, and to almost zero in the other liver. The heart muscle of the same patient showed an increased glycogen content of 3 per cent, and phosphorylase activity was almost missing. UDPGlucose --glycogen transferase was normal (cf. Mommaerts, Illingworth *et al.* [4]. this might have been the result of questionable preservation of the tissues in the deep freezer.

Attention was recently called to deficient liver phosphorylase activity in some instances of glycogen storage disease by Hers [3]. Glucose-6--phosphatase and amylo-1,6-glucosidase activity were within normal limits; muscle glycogen and muscle phosphorylase were normal. Also, in a peculiar type of glycogen deposition disease, known as McArdle's myopathy, phosphorylase was found to be missing, while UDPGlucose--glycogen transferase was normal (cf. Mommaerts, Illingworth *et al.* [4]). So far we are unable to explain precisely the phosphorylase defect in the liver. Does it consist of an insufficiency in diphospho-phosphorylase--kinase, in phosphorylase-phosphatase, or is it of an entirely different nature? Of particular interest is our second case with two enzymes missing — phosphorylase in heart and glucose-6-phosphatase in liver. This is hard to explain on the basis of the one gene — one enzyme hypothesis.

Another gross anomaly in the hepato-nephromegalic glycogenosis is the hyperlipemia and accumulation of large amounts of neutral fat and phospholipids in the liver. Most of the weight of the liver is due to this excess of fatty material, while glycogen accumulation represents only a negligible share of the total weight. It is generally accepted that the underlying mechanism is identical with that in severe Diabetes Mellitus. Fat and protein are called on to meet the normal caloric needs. The nitrogen content of the liver is markedly reduced, in one of the autopsy livers it amounted to only 1.84 mg. per cent. The partition of phospholi-

pids by phosphorus analysis (G. Schmidt *et al.* [5]) was studied in this case by G. Schmidt and showed the following results:

Total phospholipids . . . . . 140 mg. per cent,
Lecithin and cephalin . . . . . 131.7 mg. per cent,
Acetal phospholipids (plasmalogen) . . . 3.5 mg. per cent,
Sphyngomyeline . . . . . 4.8 mg. per cent,
Ratio phospholipids/nitrogen . . . . 76 (normal approx. 40),
Total lipids in the peripheral blood amounted to 7000 mg. per cent.

It is open to discussion whether or not the huge fat, phospholipid and cholesterol content of the liver obscured the measurements of phosphatase activity. If corrections were allowed for those deposits, glucose-6--phosphatase activity per 100 mg. fresh liver might approach almost normal values.

Taking all these contradictory data into account, we are far from being able to explain the hepato-nephromegalic type at least as a simple glucose-6-phosphatase deficiency. As Lowe *et al.* [7] have pointed out, most investigations in liver glycogen disease focused on the initial stages of glycogenolysis, particularly the hydrolysis of glucose-6-phosphate. In the cases with low phosphorylase activity of the liver normal glycogen synthesis catalyzed by UDPGlucose-glycogen transferase may explain the excessive accumulation of glycogen being the result of the disturbance of a physiological equilibrium. In the von Gierke type of the disease, however, no satisfactory hypothesis can be offered so far, to explain some of the unexpected findings and bridge over the wide discrepancy between *in vitro* studies and *in vivo* observations.

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BADANIA NAD PRÓBKAMI WĄTROBY POBRANYMI NA SEKCJI PRZYPADKÓW CHOROBY GLIKOGENOWEJ.

NIEZGODNOŚĆ MIĘDZY WYNIKAMI UZYSKANYMI Z DOŚWIADCZEŃ *IN VITRO* ZE SPOSTRZEŻENIAMI *IN VIVO*

Streszczenie

Inkubowanie homogenatów wątrób pochodzących z dwóch przypadków choroby glikogenowej prowadziło do powstania znacznych ilości glikozy, wystarczających do utrzymania normalnych poziomów glikozy we krwi. Przedyskutowano znaczenie tych wyników w świetle enzymatycznej patogenezy choroby glikogenowej.

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