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（検診及び豫防注射）

第十三條　都道府縣知事は、狂犬病が發生した場合において、そのまん延の防止及び撲滅のため必要と認めるときは、期間及び區域を定めて豫防員をして犬の一せい檢診をさせ、又は臨時の豫防注射を行わせることができる。

（病性鑑定のための措置）

第十四條　豫防員は、病性鑑定のため必要があるときは、都道府縣知事の許可を受けて、犬の死體を解剖し、又は解剖のため狂犬病にかかつた犬を殺すことができる。

2、前項の場合においては、第六條第七昭の規定を準用する。

（移動の制限）

第十五條　都道府縣知事は、狂犬病のまん延の防止撲滅のため必要と認めるときは、期間及び區域を定めて、犬又はその死體の當該都道府縣の區域内における移動、當該都道府縣内への移入出又は當該都道府縣外への移出を禁止し又は制限することができる。

（交通のしや斷又は制限）

第十六條　都道府縣知事は、狂犬病のまん延の防止及び撲滅のため必要があると認めるときは、厚生省令の定めるところにより、期間を定めて狂犬病にかかつた犬の所在の場所及びその附近の交通をしや斷し、又は制限することができる。但しその期間は、七十二時間をこえることができない。

（集合施設の禁止）

第十七條　都道府縣知事は、狂犬病まん延の防止及び撲滅のため必要と認めるときは、犬の展覽會その他の集合施設の禁止を命ずることができる。

（けい留されていない犬の抑留）

第十八條　都道府縣知事は、狂犬病のまん延の防止及び撲滅のため必要と認めるときは、豫防員をして第十條の規定によるけい留の命令が發せられているにかかわらずけい留されていない犬を抑留させることができる。

2、前項の場合には、第六條第二項から第七項までの規定を準用する。

（厚生大臣の實施命令）

第十九條　厚生大臣は、狂犬病のまん延の防止及び撲滅のため緊急の必要があると認めるときは、地域及び期間を限り、都道府縣知事に第十三條及び第十五條から前條までの規定による措置の實施を命ずることができる。

第四章　補　則

（公務員等の協力）

第二十條　公衆衞生又は治安維持の職務にたずさわる公務員及び獸醫師は狂犬病豫防のため、豫防員から協力を求められたときは、これを拒んではならない。

（抑留所の設置）

第二十一條　都道府縣知事は、第六條及第十八條の規定により抑留した犬を收容するため、當該都道府縣内に犬の拘留所を設け、豫防員にこれを管理させなければならない。

（手數料の費途）

第二十二條　第四條や第四項の規定により徵收された手數料は、すべてこの法律の目的達成のために用いられなければならない。

（費用負擔區分）

第二十三條　この法律の規定の實施に要する費用は、左に掲げるものを除き、都道府縣の負擔とする。

第一　國の負擔する費用

第七條の規定による輸出入檢疫に要する費用（輸出入檢疫中の犬の飼養管理費を除く。）

第二　犬の所有者の負擔する費用

一、第四條の規定による登錄の手續に要する費用

二、第五條及び第十三條の規定による犬の豫防注射の費用

三、第六條及び第十八條の規定による犬の抑留中の飼養管理費及びその返還に要する費用

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四、第七條の規定による輸出入檢疫中の犬の飼養管理費
五、第八條の規定による屆出に要する費用
六、第九條の規定による隔離及び指示により行つた處置に要した費用

（處分等の行爲の承繼人に對する効力）

第二十四條　この法律又はこの法律に基く命令の規定による處分及び手續その他の行爲は、當該行爲の目的である犬について所有權その他の權利を有する者の承繼人に對しても、またその効力を有する。

（政令で定める事）

第二十五條　この法律中　都道府縣」又は「都道府縣知事」とあるのは、保健所法（昭和二十二年法律第百一號）第一條の規定に基く政令で定める市については、「市」又は「市長」と讀み替えるものとする。但し、第八條第二項及び第三項の規定については、この限りでない。

第五章　罰　則

第二十六條　左の各號の一に該當する者は、五萬圓以下の罰金に處する。
一、第七條の規定に違反して檢疫を受けない（犬第二條の規定により準用した場合における動物を含む。以下この章中同じ。）を輸出し、又は輸入したもの
二、第八條第一項の規定に違反して犬についての屆出をしなかつた者
三、第八條第一項の規定に違反して犬を隔離しなかつた者

第二十七條　左の各號の一に該當する者は、三萬圓以下の罰金に處する。
一、第四條の規定に違反して犬の登錄の申請をせず、又は鑑札を犬に着けなかつた者
二、第五條の規定に違反して犬に豫防注射を受けさせず、又は注射濟票を着けなかつた者
三、第九條第二項に規定する犬の隔離についての指示に從わなかつた者
四、第十條に規定する犬に口輪をかけ、又はこれをけい留する命令に從わなかつた者

五、第十一條の規定に違反して犬を殺した者
六、第十二條の規定に違反して犬の死體を引き渡さなかつた者
七、第十三條に規定する犬の檢診又は豫防注射を受けさせなかつた者
八、第十五條に規定する犬又はその死體の移動、移入又は移出の禁止又は制限に從わなかつた者
九、第十六條に規定する犬の狂犬病のための交通のしや斷又は制限に從わなかつた者
十、第十七條に規定する犬の集合施設の禁止の命令に從わなかつた者

附　則

1、この法律は、公布の日から施行する。
2、昭和二十五年における第四條に規定する豫防注射は、同年九月三十日までにこれを行うように厚生省令をもつて定めなければならない。
3、家畜傳染病豫防法の一部を次のように改正する。
第一條第一項中「犬」を削る。
第四條第一項第一號中「牛疫、牛肺疫又は狂犬病」を「牛疫又は牛肺疫」に改め、同條第二項を削る。
第五條第一項中「豚ノバラチフス」の次に「狂犬病」を加える。
第十七條　削　除
第二十三條第三第五號を削り、第六號を第五號とする。
第二十四條第一項第一號但書中「犬及」を削る。
4、この法律施行前にした行爲に對する罰則の適用については、なほ從前の例による。

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◎厚生省第五十二號
狂犬病豫防法施行規則を次のように定める
昭和二十五年九月二十二日

厚生大臣 黒川武雄

狂犬豫防法施行規則

（届用のための報告）

第一條 都道府縣知事は、當該都道府縣内において狂犬病豫防法（昭和二十五年法律第二百四十七號。以下「法」という）第二條但書に規定する動物について狂犬病が發生し、同條但書の規定に基いて法の一部を準用する必要があると認めるときは、動物の種類及び狂犬病の發生の狀況を厚生大臣に報告しなければならない。

（豫防員の證票）

第二條 法第三條第二項の規定による狂犬病豫防員（以下「豫防員」という。）の身分を示す證票は、別記樣式第一による。

（登錄の申請）

第三條 每年四月一日現在において生後九十一日以上の犬を所有してゐる者は、法第四條第一項の規定によつて四月三十日迄に左の事項を記載した申請書を提出しなければならない。

一、所有者の住所及び氏名又は名稱
二、犬の所在地
三、犬の種類
四、犬の生年月日
五、犬の毛色
六、犬の性別
七、犬の名
八、犬の體格
九、前六號の外犬の特徵となるべき事項

2、四月二日から翌年三月三十一日までの間に、生後九十一日以上の犬で、登錄をされていないもの又は登錄をされてゐるかどうかが明らかでないものを所有するに至つた者は、三十日以内に其の事由を具し前項に準じて申請を提出しなければならない。

3、前二項の規定による申請に基く登錄の効力は、登錄の日の次の三月三十一日迄とする。

（原簿及び鑑札の樣式）

第四條 法第四條第二項に規定する原簿は、別記樣式第二による。

2、法第四號第二項に規定する犬の鑑札は、別記樣式第三による。

（鑑札の再交付）

第五條 犬の所有者は、鑑札を汚損し又は失つたときは、其の事由を書き、汚損した場合には、其の鑑札を添え、三十日以内に犬の所在地の市町村長（東京都の區の存する區域にあつては區長とする。以下同じ）を經て都道府縣知事に再交付を申請しなければならない。

2、前項の規定により鑑札の再交付を申請した後、失つた鑑札を發見したときは、五日以内に犬の所在地の市町村長を經てこれを都道府縣知事に提出しなければならない。

（所有者の住所等の變更）

第六條 犬の所有者は、その住所を移したとき又はその氏名若しくは名稱を變更したときは、その旨を犬の所在地の市町村長を經て、都道府縣知事に届け出なければならない。

（犬の所在地の變更）

第七條 犬の所有者は、犬の所在地を移したときは、その旨を、三十日以内に犬の新所在地の市町村長を經て都道府縣知事に届け出なければならない。

2、前項の届出を受けた都道府縣知事が犬の舊所在地の都道府縣知事と異るときは、その届出を受けた都道府縣知事は、し

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の新所在地の市町村長を経て、犬の所有者に、犬の舊所在地の都道府縣知事から交付を受けた鑑札と引替に鑑札を交付するとともに犬の舊所在地の都道府縣知事に犬の新所在地を通知しなければならない。

3、前項の通知を受けた都道府縣知事は、通知をした都道府縣知事に、その犬の原簿を送らなければならない。

（死亡及び所有權の放棄）

第八條　犬の所有者は、犬が死亡したとき又はその犬について所有權を放棄しようとするときは、鑑札及び通知済票を添えて犬の所在地の市町村長を經て都道府縣知事にその旨を届け出なければならない。但し、鑑札及び狂犬病注射済票を添えることができない正當の事由のあるときは、添えることを要しない。

（所有者の變更）

第九條　犬の所有者がかわつたときは、新所有者は、新舊の所有者の住所及び氏名又は名稱並びに犬の新舊の所在地を書き犬の新所在地の市町村長を經て三十日以内に都道府縣知事に届け出なければならない。

2　前項の場合には、第七條第二項及び第三項の規定を準用する。

（豫防注射の時期）

第十條　犬の所有者（所有者以外の者が管理する場合はその者。以下同じ）は、法第五條第一項の規定により、その犬について、狂犬病の豫防注射を四月から六月までの間及び十月から十二月までの間に各一回受けさせなければならない。

2、前項に掲げる期間以外において、生後九十一日以上の犬であつて、過去六箇月以内に狂犬病の豫防注射を受けていないもの又は受けたかどうかが明らかでないものを所有又は管理するに至つた者は、その日から三十日以内にその犬について狂犬病の豫防注射を受けさせなければならない。

（注射済票の交付）

第十一條　獸醫師が狂犬病の豫防注射を行つたときは、その犬の所有者に對して、別記様式第四による豫防注射證を交付しなければならない。

2、犬の所有者は、前項に規定する注射済證を保健所長に提示し、注射済票の交付を受けなければならない。

3、注射済票は、別記様式第五による。

（注射済票の再交付）

第十二條　犬の所有者は、注射済票を汚損し又は失つたときは、その事由を書き、注射済證を提示し、且つ汚損した場合にはその注射済票を添えて保健所長に申請して再交付を受けなければならない。

2、第五條第二項の規定は、前項の場合に準用する。

（捕獲人の證票）

第十三條　法第六條第三項の規定による捕獲人の身分を示す證票は、別記様式第六による。

（所有者への通知）

第十四條　豫防員は、法第六條第四項の規定（法第十八條第二項の規定によつて準用される場合を含む。）によつて犬の所有者に通知するときは、配達證明郵便又は使丞によらなければならない。

（處分前の評價）

第十五條　豫防員は、法第六條第六項の規定によつて犬を處分する場合又は法第十四條第二項の規定によつて犬を殺す場合はあらかじめその犬の價格について適當な評價人三人以上に評價させておかなければならない。

（狂犬病の犬の届出事項）

第十六條　獸醫師又は犬の所有者が法第八條第一項の規定によつて市町村長に届出るべき事項は、その犬の種類、生年月日、毛色、性別、名及び體格、その犬の所有者の住所及び氏名又は名稱及びにその犬の所在地とする。

（政令で定める市）

第十七條　この省令中「都道府縣」又は「都道府縣知事」とあるのは、保健所法（昭和二十二年法律第百一號）第一條の規定に基く政令で定める市については、「市」又は「市長」と讀み替えるものとする。

附　則

1、この省令は、公布の日から施行する。

2、第三條第一項の規定にかかわらず、昭和二十五年に限り、九月一日現在の犬の所有者は九月末日までに法第四條第一項の規定による登錄の申請をしなければならない。

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の新所在地の市町村長を経て、犬の所有者に、犬の旧所在地の都道府縣知事から交付を受けた鑑札と引替に鑑札を交付するとともに犬の旧所在地の都道府縣知事に犬の新所在地を通知しなければならない。

3、前項の通知を受けた都道府縣知事は、通知をした都道府縣知事に、その犬の原簿を送らなければならない。

（死亡及び所有権の放棄）

第八條　犬の所有者は、犬が死亡したとき又はその犬について所有権を放棄しようとするときは、鑑札及び通知済票を添えて犬の所在地の市町村長を経て都道府縣知事にその旨を届け出なければならない。但し、鑑札及び注射済票を添えることができない正當の事由のあるときは、添えることを要しない。

（所有者の變更）

第九條　犬の所有者がかわつたときは、新所有者は、新舊の所有者の住所及び氏名又は名稱並びに犬の新舊の所在地を書き犬の新所在地の市町村長を經て三十日以内に都道府縣知事に届け出なければならない。

2　前項の場合には、第七條第二項及び第三項の規定を準用する。

（豫防注射の時期）

第十條　犬の所有者（所有者以外の者が管理する場合はその者。以下同じ）は、法第五條第一項の規定により、その犬について、狂犬病の豫防注射を四月から六月までの間及び十月から十二月までの間に各一回受けさせなければならない。

2、前項に掲げる期間以外において、生後九十一日以上の犬であつて、過去六箇月以内に狂犬病の豫防注射を受けていないもの又は受けたかどうかが明らかでないものを所有又は管理するに至つた者は、その日から三十日以内にその犬について狂犬病の豫防注射を受けさせなければならない。

（注射済票の交付）

第十一條　獸醫師が狂犬病の豫防注注を行つたときは、その犬の所有者に對して、別記様式第四による豫防注済射證を交付しなければならない。

2、犬の所有者は、前項に規定する注射済證を保健所長に提示し、注射済票の交付を受けなければならない。

3、注射済票は、別記様式第五による。

（注射済票の再交付）

第十二條　犬の所有者は、注射済票を汚損し又は失つたときは、その事由を書き、注射済證を提示し、且つ汚損した場合にはその注射済票を添えて保健所長に申請して再交付を受けなければならない。

2、第五條第二項の規定は、前項の場合に準用する。

（捕獲人の證票）

第十三條　法第六條第三項の規定による捕獲人の身分を示す證票は、別記様式第六による。

（所有者への通知）

第十四條　豫防員は、法第六條第四項の規定（法第十八條第二項の規定によつて準用される場合を含む。）によつて犬の所有者に通知するときは、配達證明郵便又は使送によらなければならない。

（處分前の評價）

第十五條　豫防員は、法第六條第六項の規定によつて犬を處分する場合又は法第十四條第二項の規定によつて犬を殺す場合はあらかじめその犬の價格について適當な評價人三人以上に評價させておかなければならない。

（狂犬病の犬の届出事項）

第十六條　獸醫師又は犬の所有者が法第八條第一項の規定によつて市町村長に届出るべき事項は、その犬の種類、生年月日、毛色、性別、名及び體格、その犬の所有者の住所及び氏名又は名稱及びにその犬の所在地とする。

（政令で定める市）

第十七條　この省令中「都道府縣」又は「都道府縣知事」とあるのは、保健所法（昭和二十二年法律第百一號）第一條の規定に基く政令で定める市については、「市」又は「市長」と讀み替えるものとする。

附　則

1、この省令は、公布の日から施行する。

2、第三條第一項の規定にかかわらず、昭和二十五年に限り、九月一日現在の犬の所有者は九月末日までに法第四條第一項の規定による登錄の申請をしなければならない。

3、犬の所有者は、第十條第一項の規定にかかわらず、昭和二十五年に限り、その犬について法第五條第一項の規定による狂犬病の豫防注射を、九月末日までに受けさせなければならない。

別記様式第一

表　面

12cm

8cm

第　號	寫眞をはる
所屬廳 氏名 生年月日	
狂犬病豫防員の證	
昭和　年　月　日發行	

寫眞面及び豫防員の證面には、所屬廳印を押すものとする。

裏　面

この證票を携帶する者は、狂犬病豫防法により狂犬病防員の職務を行う者で、その關係條文は次の通りであります。

狂犬病豫防法抜すい

第三條　都道府縣知事は、當該都道府縣の職員で獸醫師であるもののうちから狂犬病豫防員（以下「豫防員」という。）を任命しなければならない。

2、豫防員は、その事務に從事するときは、その身分を示す證票を携帶し、關係人の求めにより、これを呈示しなければならない。

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別記様式第二

表面

14cm × 9cm

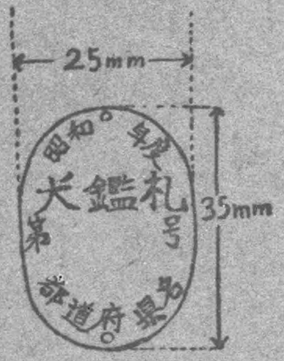
犬の原簿			
登錄年月日	登錄番號	種類	
年月日	第　號	生年月日	年月日
年月日	第　號	毛色	
年月日	第　號	性別	
年月日	第　號	名	
年月日	第　號	體格	
年月日	第　號	その他の特徴	
年月日	第　號		
年月日	第　號		
年月日	第　號		
備考			

（用紙は厚紙とする）

裏面

所有者	住所	氏名又は名稱	變更年月日	犬	所在地	變更年月日
			年月日			年月日
			年月日			年月日
			年月日			年月日
			年月日			年月日
			年月日			年月日
備老						

別記様式第三



相當堅固な金屬板とし、くびわに附着できるように穴をあける。

別記様式第四

12cm × 17cm

第　號

狂犬病豫防注射濟證

所有者（管理者）住所
氏名

種別		生年月日
毛色		性別
名		體格
その他の特徴		

右の犬に對して狂犬病豫防注射を　年　月　日に行つたことを證明します

實施者　住所
氏名　印

◎注意　本證を　保健所（保健所の所在地）に提示して注射濟票の交付を受けて下さい。

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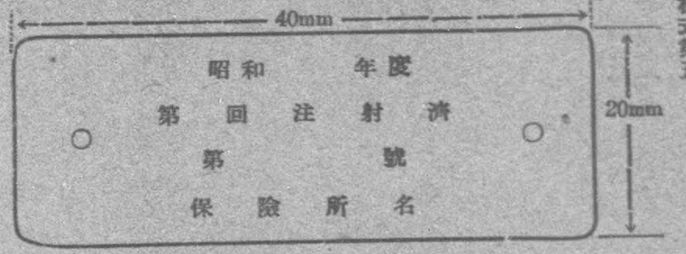
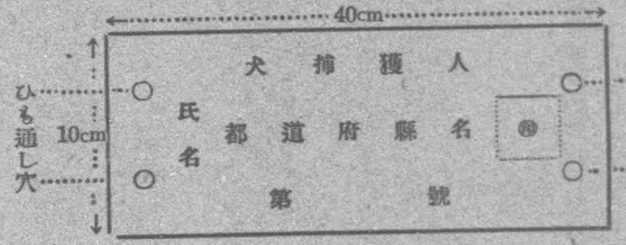
別記様式第五



相當堅固な金屬板とし、くびわに附着できるように穴をあける

別記様式第六



青色地に白字で記した腕章とする

狂犬病豫防法施行細則

（昭和二十五年十月二十三日　茨城縣規則第六七號）

第一條　犬の所有者又は獸醫師は狂犬病豫防法（昭和二十五年法律第二四七號、以下法という）狂犬病豫防法施行規則（昭和二十五年厚生省令第五十二號、以下規則という）及びこの細則の定めるところにより知事に提出する申請書及び届書の類は犬の所在地の市町村長を經て所轄保健所長に提出しなければならない。

第二條　犬の所有者が規則第三條により登録申請をしようとするときは別記様式第一號の申請書に別に定め手數料に相當する茨城縣收入證紙を添えて提出しなければならない。

第三條　犬の所有者は犬が逃離又は失踪等によつて所在が不明になつたときは、その事實が起つたときから三日以内に別記様式第二號による届け出をしなければならない。

第四條　規則第八條による犬の所有者が所有權を放棄しようとするときは正當の理由ないかぎり十五日以前に別記様式第三號により届け出なければならない。

第五條　獸醫師が狂犬病の豫防注射を行つたときは、別記様式第四號による注射報告書を翌月五日迄に保健所長に届けなければならない。

第六條　法第六條及び法第十八條の規定により捕獲した犬は保健所の犬の抑留所に抑留されなければならない。

第七條　捕獲人の指定を受けようとするものは、別記様式第五號による申請書を知事に提出しなければならない。

第八條　犬の捕獲人は規則第十三條による腕章の外別記様式第六號の捕獲人證を常時携帶し關係人の求めによりこれを呈示しなければならない。

第九條　犬の展覽會を開催しようとするもの及び犬を集合せしめる施設を使用しようとするものは十月以前に別記様式第七號により知事の許可を受けなければならない。

第十條　保健所長は次に掲げる報告書を知事に提出しなければならない。

一、畜犬關係月報

（別記様式八號）

二、狂犬病及び疑似狂犬病發生報告書、その都度（別記様式九號）

附　則

1、この細則は公布の日から施行する。

別記第一號様式

畜犬登録申請書

一、所有者又は管理者住所氏名

二、犬の所在地

三、犬の種類　犬名　年令　性別　毛色　體格　特徴

右狂犬病豫防法第四條第一項の規定により茨城縣收入證紙貳百圓を貼布して申請します。

昭和　年　月　日

茨城縣知事　殿

所有者住所

氏名　㊞

收入證紙貼布場所

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別記第二號樣式

畜犬の盜難又失踪届

一、所有者又は保管者の住所氏名
二、畜犬の種類　犬名　性別　年令　毛色　體格　特徴
三、畜犬の盜難又は失踪年月日
四、畜犬の盜難又は失踪經過
五、畜犬の盜難又は失踪場所
六、畜犬の登錄年月日
七、畜犬の登錄番號

右狂犬病豫防法に基く施行細則第三條の規定によりお届けします

昭和　年　月　日

所有者住所
氏名　㊞

茨城縣知事　殿

別記第三號樣式

畜犬登錄取消届

一、所有者又は管理者住所氏名
二、登錄年月日
三、登錄番號
四、畜犬の種類　犬名　生年月日　性別　毛色　體格　特徴
五、登錄取消年月日
六、登錄取消理由

右狂犬病豫防法施行規則第八條に基き鑑札及び注濟濟票を添へ畜犬の登錄を取消したいからお届けします

昭和　年　月　日

所有者住所氏名　㊞

茨城縣知事　殿

第四號樣式

昭和　年　月　日

保健所長殿

住所
開業地
獸醫師氏名

狂犬病豫防注射實施報告

月中に於ける標記について左記の通り報告する

注射月日	使用ワクチン名及び容量	登錄番號	濟票番號	飼養住所氏名	

二四

第五號樣式

捕獲人指定申請書

本籍地
現住所

狂犬病豫防法第六條第二號の指定捕獲人になりたいので履歷書及び健康證明書を添え申請致します

申請者氏名

氏名　㊞

茨城縣知事　殿

二五

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別記第六號樣式

表面

12cm

8cm

寫眞をはる

第　號

住所
氏名　　年令

犬捕獲人の證

捕獲地域、
年月日

茨城縣㊞

裏面

この證票を携帶するものは狂犬病豫防法第六條第三項同法第十八條第二項により豫防員の指示によつて犬の捕獲を行うものである。

その遵守事項は次の通りとする

遵守事項

一、本證は他人に貸與してはならない

二、本證は、犬の捕獲の場合は必ず携帶しなければならない

三、本證は關係人の求めがあつた場合は呈示しなければならない

四、本證の有効期限は一ケ年とする

別記第七號樣式

犬の展覽會又は集合施設の使用許可申請書

一、責任者住所氏名
二、集合期間
三、集合目的
四、集合場所
五、集合犬の登錄保健所名
六、集合犬の頭數
七、開催施設の大要

右狂犬病豫防法に基く施行細則第八條第一項により申請します

昭和　年　月　日

開催責任者住所
氏名　　印

茨城縣知事　殿

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第八號樣式

保護第　號

年　月　日

茨城縣知事　殿

畜犬に關する月報

月分標記の件左の通り報告します

畜犬現在數		
月間登錄取扱件數	登錄畜犬數	
	鑑札交付數	
	斃犬數	
	鑑札再交付數	
豫防注射施行件數	縣注射實施數	
	開業獸醫注射實施數	
	注射濟票交付數	
	注射濟票再交付數	
畜捕獲頭數	捕獲數	
	畜主返還數	
	處分數	

處分方法

保健所長

二八

第九號樣式

○○發第　號

年　月　日

茨城縣知事　殿

狂犬病及び疑似狂犬病發生報告書

標記の件左の通り報告致します

畜主住所氏名						
登錄有無	種類	犬名	毛色	特徵		
年令	體格	性別	摘要			

發病月日	死亡月日	豫防注射月日	診檢月日	診檢所場
月　日	月　日	月　日	月　日	

被害者氏名	住所	性別	年令	職業	咬傷部位	咬傷後處置

被害者氏名	畜主氏名	畜主住所	咬傷後處置

1、感染系路

保健所長

狂犬病豫防員

㊞　㊞

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2、被害狀況	
3、症　狀	
4、經　過	
5、防疫處置	
備　考	

發衛第一七〇號
昭和二十五年十月五日

茨城縣知事殿

厚生事務次官

狂犬病豫防法の施行について

さきの臨時國會において成立をみた狂犬病豫防法（昭和二十五年法律第二百四十七號）は本年八月二十六日公布施行され、又同法施行規則も本年九月二十二日厚生省令第五十二號をもつて公布施行されることになつたが、本法制定の主旨は、近年狂犬病の發生が激増し、これによる被害が甚大である狀況に鑑み本病の豫防、防疫體制を一層徹底強化するため狂犬病發生時における防疫措置を完璧ならしめるとともに、通常時においても本病の豫防　防疫體制の萬全を期するため豫防、防疫對象を確實に把握し、常時すべての犬に免疫を與へ、我が國をして狂犬病の無毒地域たらしめようとすることにあるのであり、左記事項に留意の上、その運營に遺憾なきを期せられたい、なほ本病の豫防については犬の所有者の責任自覺が第一條件であるので、新に犬の所有者に種々の義務を課した本法の施行を機として犬の所有者の啓蒙指導に持に努力せられたく命によつて通知する

記

第一、豫防注射

一、法第五條の規定による豫防注射は、原則として開業獸醫に行わせることとし、豫防員が豫防注射を行うことは、近隣に開業獸醫の居ない保健所において犬の所有者が希望する場合等とせられたい。

二、豫防注射の實施については、左の方式によられたい。

a、豫防注射の實施計畫樹立にあつては豫め地方獸醫協會等と充分協議すること

b、定期豫防注射は集合注射によることとし、その際は保健所職員が立ち合い、注射施行と同時に所有者に注射濟票を交付すること

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c、定期豫防注射漏れ、及び注射期間以外において注射の必要を生じた犬については、開業獣醫師が個々に注射を行うことを原則とすること

d、その場合、豫防注射を受けた、犬の所有者が個々に保健所へ注射済票の交付を受けに行く煩雑を避けるため、豫め開業獣醫師に注射済票を渡しておき、その交付について保健所長に報告せしめるような便法を講じても差支えない。但し、この場合には注射済票の保管及び交付について十分に監督すること。

開業獣醫師が豫防注射を行うに當つては、狂犬病豫防液の品質を充分保持し得る設備、及び器具を充分に殺菌し得る設備を整備するように指導すること。と

豫防注射済票については四、五、六月の分と十、十一、十二月の分とは、これの色彩を異にする等識別し易いようにすること。

輸入された犬であつて外國で次期の豫防注射時期まで有効な注射を受けていることを證明する書面（外國で發行した證明書は動植物檢疫所（出張所を含む、以下同じ）で保管し同所においてその寫を發行する）があるもの又は動植物檢疫所において、犬の輸入檢疫のため豫防注射を實施した旨を證明する書面のあるものについては當該書面により注射済票を交付すること

三、犬の所有者が開業獣醫師に對して支拂の注射料金については不當に高額とならぬよう豫め開業獣醫と協議して決定すること。

四、法第十三條の臨時の豫防注射の實施については可及的開業獣醫師の協力を得てこれを行うようにすること。

第二、抑犬の抑留

法第六條第一項の運用については、生後九十日未滿の犬並に犬の輸入檢疫を受けた旨を證明する書面のある犬であつて、その定着地に終付するもの、及びその後の十續を完了するのに必要な期間内のもの等正當な理由により鑑札及び注射済票を着けていない犬については所有者にこれをけい留するように指導するとともに抑留しないよう充分徹底せられたい。

抑留期間は從來よりも長期となり抑留犬數も增加する見込みであるから、早急に法第二十一條の規定により犬の抑留所の設置を計られたい。

この抑留所は各保健所管轄區域に一ケ所ずつ設置することを原則とし概ね左の基準によられたい。

一、抑留室の床は排水よく、清掃に便なこと

二、一頭ずつけい留出來る設備のあること

第三、狂犬病豫防員

狂犬病豫防員は本法施行の第一線機關として重要なものであるから、充分な活動をするため必要數を任命し各保健所毎に配置するようにせられたい。

第四、權限の委任

左記の事務は保健所法第三條の規定に基づいて都道府縣知事より都道府縣保健所長（又は保健所法第一條の規定に基く政令で定める市の市長より市保健所長に委任せられたい。

一、犬の登錄の申請を受理すること（法第四條第一項）

二、犬の原簿を保管し犬の鑑札を交付すること（法第四條第二項）

三、狂犬病發生の届出について市町村長から報告を受けること（法第八條第二項）

四、交通のしや斷又は制限を命ずること（法第十六條）

なお法第八條第二項の報告を受けた保健所長は知事に報告するようにせられたい。

第五、手數料

犬の鑑札再交付手數料並に狂犬病豫防注射済票の交付手數料及び同票再交付手數料を徵收し得るよう地方公共團體手數料令の改正について目下手續中である。

DECLASSIFIED E.O. 12065 SECTION 3-402/NNDG NO. 775013

茨城縣規則第六十七號

狂犬病豫防法施行細則を次のように定める

昭和二十五年十月二十三日

狂犬病豫防法施行細則

茨城縣知事　友末洋治

（茨城縣規則第六十七號　昭和二十五年十月二十三日）

第一條　犬の所有者又は獸醫師は狂犬病豫防法（昭和二十五年法律第二四七號　以下法という）狂犬病豫防法施行規則（昭和二十五年厚生省令第五十二號、以下規則という）及びこの細則の定めるところにより知事に提出する申請書及び屆書の外は犬の所在地の市町村長を經て所轄保健所長に提出しなければならない。

第二條　犬の所有者が規則第三條により登錄申請をしようとするときは、別記樣式第一號の申請書に別に定める手數料に相當する茨城縣收入證紙を添えて提出しなければならない。

第三條　犬の所有者は、犬が盜難又は失踪等によつて所在が不明になつたときは、その事實が起つたときから三日以內に別記樣式第二號による屆出をしなければならない。

第四條　規則第八條による犬の所有者が所有權を放棄しようとするときは正當な理由のないかぎり十五日以前に別記樣式第三號により屆け出なければならない。

第五條　獸醫師が狂犬病の豫防注射を行つたときは、別記樣式第四號による注射報告書を翌月五日迄に保健所長に屆け出なければならない。

第六條　法第六條及び法第十八條の規定により捕獲された犬は保健所の犬の抑留所に抑留させなければならない。

第七條　捕獲人の指定を受けようとする者は別記樣式第五號による申請書を知事に提出しなければならない。

第八條　犬の捕捕獲人は規則第十三條による腕章の外別記樣式第六號の捕獲人證を常時携帶し關係人の求めによりこれを呈示しなければならない。

第九條　犬の展覽會を開催しようとするもの及び犬を集合せしめる施設を使用しようとするものは十日以前に別記樣式第七號により知事の許可を受けなければならない。

第十條　保健所長は次に揭げる報告書を知事に提出しなければならない。

一、畜犬關係月報　（別記樣式第八號）

二、狂犬病及び疑似狂犬病發生報告書　（別記樣式第九號）

附　則

1、この細則は公布の日から施行する。

DECLASSIFIED E.O. 12065 SECTION 3-402/NNDG NO. 775013

別記様式第一號

畜犬登錄申請書

一、所有者又は管理者住所氏名

二、犬の所在地

三、犬の種類　犬名　年令　性別　毛色　體格　特徴

右狂犬病豫防法第四條第一項の規定により茨城縣收入證紙貳百圓を貼布して申請します。

昭和　年　月　日

茨城縣知事　殿

收入證紙貼布場所

所有者住所

氏名　㊞

別記様式第二號

畜犬の盜難又は失踪届

一、所有者又は保管者の住所氏名

二、畜犬の種類　犬名　年令　性別　毛色　體格　特徴

三、畜犬の盜難又は失踪年月日

四、畜犬の盜難又は失踪場所

五、畜犬の盜難又は失踪經過

六、畜犬の登錄年月日

七、畜犬の登錄番號

右狂犬病豫防法に基く施行細則第三條の規定によりお届けします

昭和　年　月　日

茨城縣知事　殿

所有者住所

氏名　㊞

DECLASSIFIED E.O. 12065 SECTION 3-402/NNDG NO. 775013

別記様式第三號

畜犬登錄取消屆

一、所有者又管理者氏名
二、登錄年月日
三、登錄番號
四、畜犬の種類　犬名　生年月日　性別　毛色　體格　特徴
五、登錄取消年月日
六、登錄取消理由

右狂犬病豫防法施行規則第八條に基き鑑札及び注射済票を添え畜犬の登錄を取消したいからお屆けします。

昭和　年　月　日

所有者住所氏名　㊞

茨城縣知事　殿

別記様式第四號

昭和　年　月　日

保健所長殿

住所
開業地
獸醫師氏名

狂犬病豫防注射實施報告

月中における標記について左記の通り報告する

注射年月日	使用ワクチン名及び容量	登錄番號	済票番號	飼養者住所	氏名

DECLASSIFIED E.O. 12065 SECTION 3-402/NNDG NO. 775013

別記様式第五號

捕獲人指定申請書

本籍地

現住所

申請者氏名

狂犬病豫防法第六條第二號の指定捕獲人になりたいので履歴書及び健康證明書を添え申請致します。

茨城縣知事　殿

氏名　㊞

別記第六號様式

衣面

12cm

8cm

第　號

寫眞をはる

住所

氏名

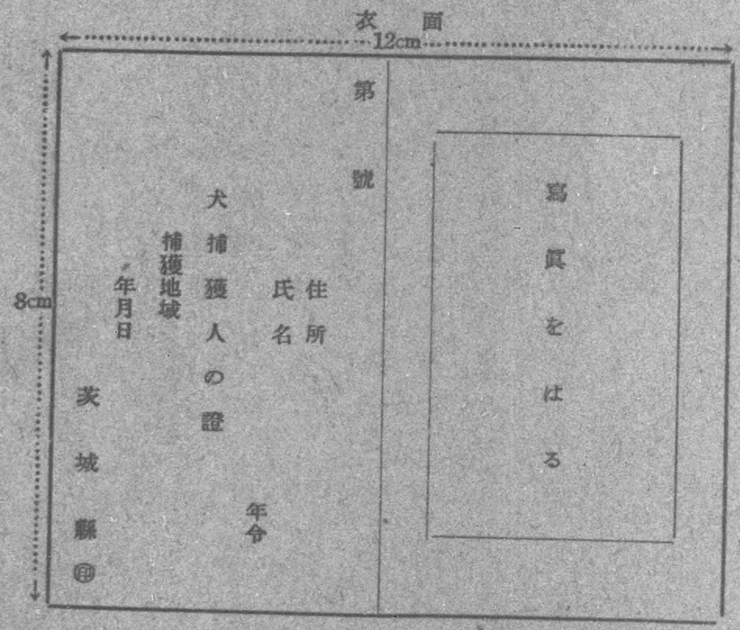
年令

犬捕獲人の證

捕獲地域

年月日

茨城縣㊞



裏面

この證票を携帶するものは狂犬病豫防法第六條第三項同法第十八條第二項により豫防員の指示によつて犬の捕獲を行うものである。

その遵守事項は次の通りとする

遵守事項

一、本證は他人に貸與してはならない

二、本證は、犬の捕獲の場合は必ず携帶しなければならない

三、本證は關係人の求めがあつた場合は呈示しなければならない

四、本證の有効期限は一ヶ年とする

DECLASSIFIED E.O. 12065 SECTION 3-402/NNDG NO. 770013

別記様式第七號

犬の展覽會又は集合施設の使用許可申請書

一、責任者住所氏名
二、集合期間　自
　　至
三、集合場所
四、集合目的
五、集合犬の登錄保健所
六、集合犬の頭數
七、集合施設の大要

右狂犬病豫防法に基く施行細則第九條第一項により申請します。

昭和　年　月　日

茨城縣知事　殿

開催實任者住所
氏名　㊞

別記様式第八號

○○發第　號

年　月　日

茨城縣知事　殿

保健所長

畜犬に關する月報（　月分）

標記の件左記の通り報告致します。

畜犬現在數	登錄畜犬數		頭
	鑑札交付數		枚
	廢犬數		頭
	鑑札再交付數		枚
月中登錄取扱件數	豫防注射實施件數		頭
	開業獸醫注射實施數		頭
	注射濟票交付數		枚
	注射濟票再交付數		枚
犬捕獲頭數	捕獲數		頭
	注主返還數		頭
	處分數		頭

（處分方法）

DECLASSIFIED E.O. 12065 SECTION 3-402/NNDG NO. 775013

茨城縣衛生關係試驗免許營業許可等手數料徵收規則

（昭和二十五年十月二十五日茨城縣規則第七一號）

茨城縣衛生關係試驗免許營業許可等手數料徵收規則を次のように定める。

茨城縣衛生關係試驗免許營業許可等手數料徵收規則（抜萃）

第一條の四十二　家畜の檢疫免疫血清及び豫防液の注射並びに藥浴手數料　百圓
四十三　犬の登錄手數料　參百圓
四十四　犬の鑑札再交付手數料　五十圓
四十五　狂犬病豫防注射濟票再交付手數料　三十圓

第二條　この規則に定める手數料は茨城縣收入證紙によつて納付しなければならない。

第三條　徵收した手數料は何等の事由があつてもこれを還付しない。

附　則

1、この規則は公布の日から施行し昭和二十五年九月一日から適用する。

2、犬の登錄手數料は昭和二十六年三月三十一日までは二百圓とする。

輕犯罪法（昭和二十三年五月一日法律第三九號）

輕犯罪法をこゝに公布する。

輕犯罪法（抜萃）

第一條の十二　人畜に害を加える性癖のあることの明らかな犬その他の鳥獸類を正當な理由がなくて解放し又はその監守を怠つてこれを逃がした者。

第二條　前條の罪を犯した者に對しては情狀により、その刑を免除し又は拘留及び科料を併科することが出來る。

第三條　第一條の罪を敎唆し、又は幫助した者は正犯に準ずる。

附　則

この法律は、昭和二十三年五月一日からこれを施行する。

DECLASSIFIED E.O. 12065 SECTION 3-402/NNDG NO. 775013

通牒

公衛發方二七五號
昭和二十四年七月十五日
大城縣醫師會長殿

茨城縣衛生部長

狂犬病豫防注射及び咬傷部位の處置について

一、狂犬病豫防注射

狂犬病の病狀を明瞭に呈する犬の咬傷を受けた場合、局所の處置と同時に成るべく速かに狂犬病豫防注射を實施することは勿論であるが、狂犬病の疑の輕度な犬によつて下半身咬傷を受けた場合は、咬傷犬を抑留してその臨床診斷が二週間以内に決定出來るから、先づ局所の處置を施し咬傷犬の診斷を待つて豫防注射を着手しても時機を失する虞れはない。然し狂犬病の疑ひ濃厚で咬傷の深いもの、中樞に近いもの（頭部咬傷）神經の多い部分の咬傷（手指末端）等は感染の危險大であるから、咬傷犬の臨床診斷を待たずに直ちに豫防注射を行ひ、若しその咬傷犬が狂犬病でなければ豫防注射は中止してもよい。以上何れの場合に於ても、咬傷犬の早期鑑定のため咬傷患者又は診察した醫師は、その旨を速かに所轄保健所長へ報告するように御指導を願いたい。

狂犬病ワクチンは、從來傳染病研究所から製造發賣せられているが現在關東地方の需要增加のため入手に多少困難を伴ふので當部に少量であるが常備しているから、緊急の場合は當部公衆衛生課へ連絡せられたい。尚狂犬病の犬による咬傷患者に對する豫防ワクチンは縣から別記により交付するから所轄保健所長に連絡の上被害者負擔輕減に協力願いたい。

二、犬の咬傷處置について

狂犬病の疑のある犬の咬傷を受けた時の局所處置としては、咬傷部を直ちに局方發煙硝酸又は一〇%苛性加里液で腐蝕するか「パクレン」で燒灼し沃度丁幾を塗布した後繃帶を施す。

狂犬病々毒は抵抗力甚だ強く通常の創傷處置では効果がないから以上の局所處置を周知せしめられたい。

公衛發第一二〇號
昭和二十四年七月二十日

各保健所長宛

衛生部長

人體用狂犬病ワクチンについて

右について七月十五日公衛發第一七五號を以つて通牒した通り現在五人分のワクチンを公衆衛生課に常備してあるから必要の傷合には別記樣式申請書によつて交付するから公衆衛生課長宛請求せられたい。

尙ほ保健所又は市町村が直接購入して豫防注射を實施した場合に於ても前記申請書を速かに公衆衛生課長に提出せられたい。

DECLASSIFIED E.O. 12065 SECTION 3-402/NNDG NO. 775013

別記様式

人體用狂犬病ワクチン 無償 有償 交付申請書

一、豫防接種者の住所氏名　　生年月日

二、野犬畜犬の別（畜犬の場合は畜主の住所氏名）

三、犬にかまれた日時

四、犬にかまれた場所

五、咬傷部位及び程度

六、咬傷犬檢診の結果

右の者狂犬病豫防接種々を必要と認めるので該ワクチン 有償 無償 交付申請致します

年　月　日　　保健所長氏名　　印

公衆衛生課長殿

公衛發號外

昭和二十四年七月二十日

各保健所長宛

公衆衛生課長

人體用狂犬病ワクチン取扱方について

右について七月二十日公衛發第一二〇號により衛生部長名を以つて通牒せられた所であるが、該ワクチン必要の場合には保健所長の申請書を持參すれば當課に於て無償交付するから御承知ありたい。

DECLASSIFIED E.O. 12065 SECTION 3-402/NNDG NO. 775013

公衛發第一五九號
昭和二十四年八月十二日

各保健所長宛

衛生部長

人體用狂犬病ワクチン無償有償交付要領變更について

右については縣豫算其の他の都合により今後左記の場合にのみ無償交付することに變更したから、これが取扱ひについては遺憾なきよう致されたい。

尙接種を要する場合は七月十五日附公衛發第二七五號茨城縣醫師會長宛通知した狂犬病豫防注射及び咬傷部位の處置について「號一」を參照して適確なる判斷を下し指導されたい。

記

一、浮浪犬及び野犬の場合
1、咬傷犬を捕獲して臨床診斷をなし若しくは撲殺して顯微鏡的檢査或は動物試驗の結果陽性と決定したる場合
2、咬傷犬が狂犬病の疑があつて逃走又は行方不明となつた場合

二、畜犬の場合
1、咬傷犬が臨床診斷若しくは顯微鏡的檢査或は動物試驗の結果陽性と決定したる場合は畜主に該ワクチン費を負擔せしめるのを原則とするが被害者が多數發生した場合或は畜主が經濟的に負擔できない場合に限つて無償交付する

DECLASSIFIED E.O. 12065 SECTION 3-402/NNDG NO. 775013

狂犬病予防はどうしたらよいか

茨城縣衛生部公衆衛生課
保健所
市町村役場

DECLASSIFIED E.O. 12065 SECTION 3-402/NNDG NO. 775013

Reg. Record to be kept by H.C.

犬の原簿

登録年月日	登録番号	種類	
年 月 日	第 號	生年月日	年 月 日
年 月 日	第 號		
年 月 日	第 號	毛色	
年 月 日	第 號	性別	
年 月 日	第 號	名	
年 月 日	第 號	体格	
年 月 日	第 號	体格	
年 月 日	第 號	その他の特徴	
年 月 日	第 號		
年 月 日	第 號		
年 月 日	第 號		
備考			

DECLASSIFIED E.O. 12065 SECTION 3-402/NNDG NO. 775013

狂犬病豫防注射濟證

第　　号

所有者（管理者）

住所　茨城県　郡　市　町　村　番地

氏名

種類	毛色	名	その他の特徴	生年月日	性別	体格

右の犬に對し狂犬病豫防注射を　年　月　日に行つたことを證明します

住所　茨城県　郡　市　町　村　番地

実施者　氏名　㊞

●注意　本証を　保健所（保健所々在地）に提示して注射済票の交付を受けて下さい。

催告書

狂犬病豫防法第五條に基き貴市町村にて狂犬病豫防注射を實施したが接種洩れであるから左記の通り催告する。

記

一、日時　昭和　年　月　日

二、場所

注意

一、疾病その他の理由により注射猶豫の畜犬は獸医師の診斷書を添へ當該市町村長を經て狂犬病予防員に届け出ること。

二、注射に來るときは催告書を持參すること。

三、正當な理由なくして注射を受けないときは狂犬病予防法第二十七條によつて參萬円以下の罰金に処せられる。

茨城縣知事

殿

郡
市　　町
村

DECLASSIFIED E.O. 12065 SECTION 3-402/NNDG NO. 775013

通知書

茨城縣知事

殿

狂犬病豫防法第五條に基き左記の通り狂犬病豫防注射を施行する

記

一、日時　昭和　年　月　日

二、場所　　郡　　町
　　市　　村

三、注射は無料

注意

一、疾病その他の理由により注射猶予の畜犬は当該市町村長を經て狂犬病予防員に届出ること。

二、注射に來るときは通知書を持參すること。

三、正當な理由なくして注射を受けないときは狂犬病予防法第二十七條によつて參萬円以下の罰金に処せられる。

DECLASSIFIED E.O. 12065 SECTION 3-402/NNDG NO. 775013

DECLASSIFIED E.O. 12065 SECTION 3-402/NNDG NO. 77503

狂犬病は恐ろしい病氣ですが

必ず絶滅出來ます!!

それは
- 第1に　総ての犬は必ず年2回予防注射を受けること。
- 第2に　野犬、放浪犬を絶滅させる。
- 第3に　犬の繋留命令が出たら必ず繋いで置くこと。

然しこれはどうしても県民の皆様の御協力がなくては出来ません。

県では今までいろいろと狂犬病予防について皆様にお知らせや御協力をお願いをしているのですがいざ狂犬病予防になるとなかなか出来ません。

此の度の予防接種には洩れなく受けて狂犬病絶滅に心から御協力をお願いします。

狂犬病の豫防はどうすればよいか

(1) **犬を飼つたら必ず登錄しましよう。**

犬が生れてから91日になれば必ず飼主は市町村長を經て知事に登錄しましよう。

今度新しく狂犬病予防法が公布されましたので届け出をしないと3万円以下の罰金に処せられます。

最近県下には凡そ7万頭の犬がいると言われています或はそれ以上いるかも知れません、その内登錄の済んだものは60.200頭でありまして未だに沢山の無登錄犬や野良犬がいてそれらをもとに狂犬病大流行の原因となりうるものです。

(2) **豫防注射は必ず受けましよう。**

狂犬病予防注射をしない犬から発生します、昨年以来登錄の犬は全部注射を受けたが尚約1万頭の未接種の犬が居る筈ですこんな状況では狂犬病が益々増加するばかりで決して撲滅は出来ませんもし家の事情で指定の期日に役場で注射を受けられない場合は最寄の開業獸醫師で洩れなく一年に2回予防注射を受けるように致しましよう。

(3) **犬は必ずつないで置きましよう。**

狂犬は狂犬病の犬に咬まれなければ起りませんから狂犬に咬まれない様に犬をつないで置きましよう。盜難予防のため犬を飼つていても放して置くと犬はどんな人にも馴れてしまつて吠えなくなるものですその意味からもつないで置くように致しましよう。

運動の時にも危險をさけるため努めて口輪、つなをつけて引き運動をすべきです。

(4) **犬の様子に絶えず注意しましよう。**

常に犬の様子に注意して少しでも異常があつたらもよりの保健所か又は開業獣醫師に見てもらうようにしましよう。

その結果若し狂犬病の疑があることが分つたらすぐもよりの保健所へ届け出て下さい、届け出があると早速保健所の專門の獣醫が狂犬病かどうか診断に参います。

尙狂犬病の疑いがある時は必ず嚴重につないで置きましよう。

(5) **野犬をとる邪魔をしないことにしましよう。**

県では野犬の捕獲をして居ます。野良犬が可愛想だからと言つて逃したり、かくしたりしないようにしましよう。

無登錄の犬にも拘らず捕獲を邪魔したり犬の差出しをこばんだりする人が今でもまだあります狂犬病予防という公共福祉にそむくばかりでなく、一部公務執行の妨害ともなります無登錄犬が附近のお宅に居りましたら、お互に狂犬病を防ぐ為にその登錄をすることをすすめましよう。

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TREATMENT OF WHOOPING COUGH BY COMBINED VACCINATION OF H. PERTUSSIS VACCINE AND PURIFIED VACCINIA LYMPH (1)

By Hidetake YAOI
National Institute of Health of Japan, Tokyo.

Previously the author reported on a new observation that the formation of antibodies is strikingly accelerated and augmented by the combined vaccination of ordinary bacterial vaccines or toxoids and purified vaccinia virus. By applying this method to the treatment of infectious diseases, a marked effect, especially in pertussis, was observed as evidenced by the following observations:

Experimental

(1) Results of trial upon 584 patients, (January 1939 to July 1941):

Ordinary heat-killed pertussis vaccine (1.5 mg/cc) in doses according to the direction, mixed with 0.2 or 0.4 cc of purified vaccinia lymph (1:10,000) in the first dose, was used throughout the experiment. When the treatment was commenced within 2 weeks of illness, paroxysms would stop in about 10 days. But when the treatment was started after 3 or more weeks, a little longer periods were needed, although the effect varied from case to case.

The days required for cure seemed to differ in no way whether the treatment was given on non-vaccinated or vaccinated (against smallpox) children. Some cases were cured by the vaccinia virus alone. In about 30 per cent of the total cases, an extraordinary rapid cessation of paroxysms was recognized following the first injection. With 3 or 4 doses, about 80 per cent of the treated children were cured. Consequently, further injections are recommended in the persistent cases. In the most severe case, 12 doses were given, each combined with 0.2 or 0.3 cc of vaccinia virus, every other day, resulting in cure in 3 weeks.

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Table 1. Results of treatment of whooping cough by combined vaccination of H. Pertussis vaccine and purified vaccinia lymph.

No. of patients	Ages	Disease days before treatment	Interval between 1st and final injections	Doses	Vaccinal reaction (induration under skin)	Curative effect
1	8 yrs.	2 weeks	10 days	3	—	Marked effect.
2	9 months	?	7 d.	3	+	Complete cure
3	5 y.	2 w.	8 d.	3	—	Marked effect
4	2 y.	20 days	13 d.	6	—	Complete cure
5	2 y.	8 w.	27 d.	7	—	〃
6	4 y.	3 d.	6 d.	3	—	〃
7	6 y.	1 w.	7 d.	3	—	〃
8	?	4 w.	8 d.	3	—	〃
9	4 y.	1 w.	10 d.	6	—	〃
10	8 y.	4 w.	5 d.	3	—	〃
11	5 y.	5 d.	10 d.	4	—	〃
12	3 y.	5 d.	5 d.	3	—	〃
13	4 y.	4—5 d.	5 d.	3	—	〃
14	4 y.	1 w.	12 d.	6	—	〃
15	2 y.	2 w.	10 d.	3 (X)	+	〃
16	4 m.	1 w.	16 d.	6 (XX)	+	〃
17	5 m.	5—6 d.	6 d.	3	+	〃
18	8 y.	4—5 d.	5 d.	3	—	〃
19	5 y.	10 d.	11 d.	6	—	〃
20	4 y.	6 w.	40 d.	9 (XXX)	—	〃
Average			10.5 days	4.3 times		

Notes: (X) Purified vaccine lymph only, 0.2, 0.3, 0.4 cc.
(XX) 1st injection with purified vaccine lymph, 2nd to 6th injections with pertussis vaccine.
(XXX) First 5 doses with purified vaccinia lymph, another 4 doses with pertussis vaccine.

(2) Results of the further experience:

In recent experience, the injection was made either daily or every other day, 0.2 or 0.3 cc of the purified vaccinia virus having been added to the first, second and third doses respectively. The injections were repeated, if necessary, using H. pertussis vaccine alone. These measures seemed to shorten the period of the disease consistently.

(3) Results of prophylactic vaccination by means of the combined vaccines:

The vaccination was done in 5 doses, mixing the purified vaccinia virus in the first and third doses. Among 124 cases so treated in 1940, 9 cases (7%) contracted mild pertussis after from 8 months to one year and 6 months. But in each case the prophylactic effect of the former vaccination was evident, the manifestations being very mild with cough only.

(4) Some basic studies on the mechanism of the marked curative and prophylactic effect:

a) Formation of agglutinin and other immune bodies were caused in abundance by the present method of combined vaccination.

b) A marked desensitizing or antiallergic action, caused by living vaccinia virus,

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was demonstrated by means of the ordinary anaphylactic shock test on guinea pigs. It was found that when guinea pigs, sensitized with a small dose of normal horse serum, were treated with a small amount of the purified vaccinia virus prior to the provocative dose of normal horse serum, they escaped from shock and subsequent death.

If this preliminary treatment was performed 3 hours before the second dose of the serum, 0.1 cc of the purified vaccinia virus sufficiently inhibited the appearance of anaphylactic shock. But in case the preliminary treatment was performed 24 hours prior to the second dose, it would require a larger dose to produce the same effect. The above findings, especially the latter seems to explain the mechanism of the extraordinary prompt cessation of the paroxysms, observed in about 30 per cent of the treated cases.

Table 2. Desensitizing effect of purified vaccinia virus.

Amount of purified vaccinia virus	0.1 cc	0.1 cc	0.2 cc	0.3 cc	0.4 cc	0.5 cc	Control
Interval	3 hrs.	24 hrs.	do.	do.	do.	do.	do.
1	+ X	### X	### X	### X	### X	+ X	### X
2	– O	### X	### X	### X	### X	+ X	### X
3	– O	### X	### X	## X	## X	+ X	### X
4	– O	### X	### X	## X	– O	– O	### X
5	– O	### X	### X	– O	– O	– O	### X
6	– O	### X	– O	– O	– O	– O	### X
7	– O	– O	– O	– O	– O	– O	### X
8	– O	– O	– O	– O	– O	– O	## X
9	– O	– O	– O	– O	– O	– O	## X
10	– O	– O	– O	– O	– O	– O	## O
Death rate	10%	60%	50%	40%	30%	30%	90%

Notes: X death
O surviving
###, ##, + intensity of shock.

Addenda:

On the curative effect of the combined vaccination of typhoid vaccine and purified vaccinia virus:

The ordinary typhoid-paratyphoid (A and B) vaccines were used in gradually increasing doses, and purified vaccinia virus was added according to the following schedule; 0.2 cc, 0.2 cc, 0.3 cc, 0.3 cc, 0.3 cc, 0.5 cc, 0.5 cc, 0.7 cc, 0.7 cc. The evaluation of the effect, made mainly on the basis of fever and general symptoms, was divided into 3 types;

A –: apparently cured, B—: improved, C—: not improved

Those patients, whose fever dropped by lysis within a few days or within 10 days at most from the start of the treatment, indicating the definite shortening of the disease as compared with the control cases, were grouped in A-type.

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According to this criterion, 37 out of the 72 treated cases were found belonging to A-type (31 typhoid cases, 6 paratyphoid cases). In these cases, the duration of the disease was shortened by 10 days as compared with the 50 control cases treated by the ordinary method.

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STUDIES ON DENGUE (RESUME)

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1. Animal Experiment.

Since the early work of Graham (1903), the experimental studies of dengue have been carried out entirely upon human beings, needless to say, owing to the lack of suitable animals. But, apparent infection in lower animals, especially mouse, was observed by the authors at the outbreak in Osaka and Kobe in 1942.

First of all we attempted to inoculate mice, but did not forget the use of the fertilized chicken eggs at the same time to make sure of isolating the virus. In some instances, the last method apparently proved helpful.

The blood was taken within 24 hours from the onset of fever and using its serum, we isolated 6 strains of virus from 8 materials, termed as Fukuda, Takaki, Kawasaki, Kimura, Warabioka and Okajima.

We first succeeded in the inoculation of animals by the use of especial young mice, following our own experiences in the isolation of Lymphogranuloma inguinale or measles-virus, in which susceptibility was found to be very marked in the young animals.

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The use of especially young mice may, we believe, have been a main reason of our success in the isolation of the present virus. Intracerebral injection was used throughout the experiments, but from time to time other routes, such as subcutaneous or intraperitoneal injections were also tried.

Apparent infection of other animals such as young guinea pig, rabbit, rat and monkey, was also attained, when we used the mouse-virus.

General symptoms of infected mice:

Infection and incubation periods were, as a matter of course, inconstant in the early stage of isolation. The incubation period varied from several to 17 or 18 days. Even the longer incubation periods than 25 days were also seen, occasionally.

The tendency of adaptation, that is, the short and constant incubation period as usually seen in other fixed viruses, has been observed following passages through many generations of mice.

The virus-fixé, obtained after many passages is capable of killing the adult mice of 15—16 grams under apparent infection within 4 or 5 days.

Although there exists certain divergency in the symptoms according to the individual strains or grade of adaptation, general findings among the strains, nearly or completely adapted to the mouse, may be stated as follows:—

(1) Brain symptoms.

Symtoms set about in rapid cases after 2 or 3 days, in most cases 4 or 5 days and rarely 2 weeks. There first appears paralysis on legs, the mouse reeling along, losing balance and lying on their belly. Further, some animals shrink round their body, irritable, tremble with hair standing on end. And, when the sickness proceed, the mice roll about on floor and soon succumb (chronic cramp).

(2) Swelling on legs.

In some instances, the swelling appears on legs and when this symptom comes over the whole legs, the motion of mouse becoming very slow owing to the consequent paralysis, and it dies soon after.

The swelling is caused readily by a certain strain of virus but no readily by others.

At times, slight cyanosis appears on legs.

(3) Bloody excrements or haematouria are seen at times.

(4) Conjunctivitis is found rather often; the eyes are closed by the doposition of a lot of discharge.

(5) Dyspnoea, asthenia, decrease of body-weight, etc.

Autopsy findings:

(1) The brain is seen congested passably in many cases.

(2) Congestion of lung (interstitial pneumonia) is seen in almost all cases, although the grade of which is so variable as to vary from the liver-like total affection to punctated purpura or beginning blush.

(3) Often liver appears yellowic-white or its normal brick-colour faded (fatty

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degeneration, necrosis).

(4) In kidney oedematous swelling and discoloration as in the case of liver, is occasionally seen (glomerulum being injured).

(5) Suprarenal body is congested in about 50 per cent of cases.

(6) Certain pathologic change in heart (myocarditis).

(7) Spleen is mostly normal in appearance.

(8) Pustule or abcess on the organs, as lung, heart, liver, kidney, etc. (extensive infiltration composed mainly of leucocytic cells).

The pustular change is, however, found only in very rare cases.

Susceptibility of other animals to dengue:

Results of inoculation of the mouse-virus are as follows:—

(1) Guinea pig is killed by the intracerebral injection and we are able to pass the infection through several generations.

(2) Also rat (house and albino) can be infected in the same way, if inoculated intracerebrally or subcutaneously.

(3) Rabbit dies if inoculated by intracerebral or intraocular route. But, we failed to transmit the virus in a series even by means of the intracerebral injection of the brain-tissues.

(4) Monkey, inoculated with virus subcutaneously, showed a febrile reaction of about 40°C, leucopenia (3000), gingivitis, etc.

It was found, however, that by means of the intracerebral inoculation, the same animal is infected more apparently and dies after 3 or 12 days' incubation period. Owing to the paralysis of legs the infected monkey appears really a "dandy" itself.

At the autopsy, slight pneumonia and fatty degeneration of liver, were detected. The pathologic change in the suprarenal gland or kidney was not so marked, in that, it resembled the pictures usually seen in the mice inoculated with virus, not yet fully adapted to the mouse. We were able to pass the infection through 5 generations.

2. Cultivation of Dengue-Virus In-Vitro.

a. Maitland-Maitland's method:—

Dengue virus, which was passed through mice for 11 generations, was cultivated in a flask, containing Tyrode's solution and finely minced brain of chicken embryo (10—12 days' incubation) for 10 generations.

The same result was obtained also by means of minced whole embryo incubated 8 days.

Transmission of virus to the next flask, was performed suitably every 5 days. The pathogenicity of cultivated virus on mouse did not decrease by the prolonged in-vitro cultivations.

b. Goodpusture's method:—

Although the lesions on the chorio-allantoic membrane characteristic to dengue as described by Shortt and others was hardly observed, this method found especially suitable in the cultivation of the present virus.

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Furthermore, the method seemed to help the adaptation to mice as it was observed in the case of Fukuda, Warabioka and Okajima strains. Namely, the virus used to become so virulent as to kill the embryo within 4 or 5 days, when cultivated on chorio-allantoic membrane for 4 or 5 generations. Moreover, the virus virulent to the embryo, appeared also to infect the mouse more readily.

The chick embryo, inoculated with a large dose of virus dies within 4 days, accompanied by the haemorrhage or bloody spots upon the whole surface of the body.

The haemorrhagic change mentioned above seems to be more characteristic to dengue than the cloudy or whity spots on the membranes as pointed out by Shortt and others. So far, although a whity spot or turbidity on the membranes is seen also in the case of measles, but haemorrhage in the site of embryo is not so marked.

Two strains (Warabioka and Okajima) have hitherto been cultivated solely upon chorio-allantoic membranes for 99 generations (19/XI, 1944).

3. Particle Size of Dengue-Virus and Some Physical Influences on It.

(1) Diameter of dengue-virus (mouse-virus) was estimated as 15—23 mμ (19 mμ in an average), by means of the ultrafiltration of Elford. The size precisely coincides with that of yellow fever-virus as actually demonstrated by Bauer.

(2) Dengue virus differs much from ordinary filterable viruses in its low resistance to the action of phenol; the virus, suspended in 0.5% phenol solution shows certain attenuation after 24 hours, and is almost completely inactivated after 5 days, all at room-temperature of 23 or 27°C.

The same virus, when kept in a cold-room (6—8°C), maintains its original titre for 48 hours, but certain drop in titre is observed after 5 days. The control virus, without phenol, was fully virulent even after 7 days.

(3) The virus is partially inactivated at 56°C for 5 min. and completely destroyed after 30 minutes at the same temperature.

(4) The virus is inactivated by the exposure to supersonic waves.

(5) Ultraviolet-light also inactivate the virus.

(6) The virus is affected by X-rays. Although the inactivation is not yet marked at 25,000 r., it is highly damaged by 50,000 r. and completely by 100,000 r.

The completely inactivated virus thus obtained was found to have a very potent antigenic properties.

4. On the Action of Immune Sera.

Sera taken from dengue patient have certain amount of neutralizing antibody, but it cannot be said to be sufficient for practical purposes.

The immune serum, which was prepared by the repeated injections of the infected mouse brain into rabbits, proved that it contained very large amount of antibodies.

The authors herewith, could establish not only the identification of many strains but also detect the probability of the prophylaxis and therapy of dengue by means of sera. Furthermore, the side-by-side immunization was proved useful in the immunization of animals.

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Results of application of immune sera upon patients:

Encouraged by the experimental success mentioned above, the authors have prepared the immune serum using goats and a horse. We had an opportunity of testing its effect upon dengue patients at the second epidemic of Osaka (1943).

So far we could try our immune sera upon 12 patients, together with 2 patients, which received normal horse serum as control.

The results were as follows:—

Certain therapeutic effect was observed if it was used within 72 hours after the onset of fever.

It appeared especially effective when it was used at the early stage and in larger doses, as a matter of course.

The immune sera, however, seemed inert contrary to our expectation, to the fever, allowing the typical saddle-like fever in about 50 per cent of cases. But, notwithstanding the general conceptions that the pains suffered by the patients at the second rise of temperature is more severe, the patients treated with immune sera, were found to be much more relaxed and feeling merely feverish against the high temperatures.

If we calculate a "level of significance" after the method of direct probability of R. A. Fisher, estimating as "effective", when the recovery of appetite and the subsiding of the general pain were evident before 3rd day of disease, it being smaller than 0.05, sufficiently indicates the more effectiveness of immune sera than the normal horse serum.

To sum up, if immune serum, was applied to patients appropriately, although it by no means appreciably affect the fever-curve, it evidently shorten the duration of disease, that is to say, it hasten the recovery of appetite, decrease the head-ache and other pains.

Further, leucopenia is once reversed to normal although for a short duration of time. Normal serum exerts no effect on the leucocyte counts and general pains.

Serum therapy was repeated at Batavia and Bandong in 1944 to obtain the similar results. Namely, leucocyte counts returned once to normal, followed by the betterment of appetite and recovery of asthenic states.

5. The pathogenicity of Mouse-Brain Virus on Man and Its Immunogenic Properties.

Eight volunteers including the authors were inoculated with 2 representative strains, one already fixed to mouse (Fukuda) and the other still on the way to adaptation (Kimura or Warabioka). Heart-blood of infected mouse in varying doses of 0.05cc to 2.5cc was injected intradermally to the above volunteers:—

Out of these 8 volunteers, from case 1 to 6 contracted dengue very atypically and case 7 almost typically. In the formar 6 cases, temperature was found very low in general, including afebrile cases (cases 5 and 6).

The fact that the rash, if appeared, did localized at the site of injection, is noteworthy.

Case 8, which was inoculated with 2.5cc amount of blood of mouse infected with

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Kimura-strain, developed a typical dengue.

Further, in view of the general pains, the pathogenicity on man appears very closely connected with the virulence to the mouse or adaptation-grade of the strains used.

For instances, the symptom is apparently mild when volunteers were inoculated with Fukuda strain, which is completely adapted to mouse.

On the contrary, it was found that the pathogenicity upon human being is still sufficiently remaining, in the case of 2 other strains (Kimura and Warabioka), which as yet not completely fixed to mice.

Next, as regards the leucopenia, in every cases with the exception of case 8, it was not so marked, but increase in number of the young neutrophilic cells, or the so-called "shift to the left" and plasma cells were observed constantly.

Among all blood change, however, the rapid increase of the eosinophilic cells, as 4% at the lowest and 14% at the highest; 400—1,000 cells were counted absolutely — was thought as the most characteristic.

To sum up, the pathogenicity of our mouse-virus upon human beings becomes low in an inverse proportion to its adaptation-grade on mouse.

Further, regarding the blood picture, especially, in the point of eosinophiles, our mouse-virus apparently differs from the ordinary dengue.

However, 2 out of 12 cases which were investigated by the authors at Singapore, eosinophilie was very marked at the beginning of the disease, though, and it was especially marked in a reinfection case.

Immunity:

After 10 or 32 days, these experimentally induced dengue cases mentioned above were reinoculated with Kimura-strain which is still virulent for man as already described elsewhere in this paper. Two out of five persons tested were found to be refractory, and the remaining 3 persons contracted dengue, though mildly, but all found to be afebrile and showed no rash. A control (case No. 7) inoculated with the same test virus fell ill with dengue with fever of 38.1°C, rash, headache, and lumbago, etc.

With these findings, it was confirmed that the immunity develops through the infection and that it takes more than a month to reach the height.

To sum up, our mouse-virus is apparently attenuated and yet certain amount of immunity is caused by its infection. Our prophylactic measures are now going to develop based on this finding.

Several persons have hitherto been vaccinated at their departure to the southern countries.

A party including 2 girls went to the southern countries and the members fell ill with dengue one by one. A girl also contracted dengue at Macassal 4 months after the vaccination under a very slight fever of about 37.5°C. Rash was typical, sufficient to know that she was really attacted by dengue. Another girl and a gentleman have not at yet been attacked by dengue.

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6. On the Result of Prophylactic Vaccination against Dengue performed in Java.

Okajima or Fukuda strain was used in the preparation of vaccine.

Brain of young guinea pig or monkey, infected with the above mouse-virus, being ground in a motar, added with normal saline solution to make it 20% emulsion, centrifuged, and the supernatant fluid thus obtained, was used as the prophylactic vaccine.

In addition, a formalized vaccine was prepared adding the formalin in 0.15% to the above vaccine and incubating at 37°C for 24 hours.

The vaccines were injected subcutaneously in one or 2 doses in this case.

The reactions were apparently milder as compared with the previous experiences, when the virus-material (mouse-blood) was inserted intradermally.

Namely, almost no recognizable reaction was found at the 1 dose, but at the injection in 2 doses, 7 or 10 days after the first injection, localized red area appeared at the site of injection, and almost all people complained of passably severe itching. This local reaction may be said to be the main complaint of many vaccinants.

In some individuals, however, temporary head-ache, heavy feeling in the head, fatigue or swelling of the axillary lymph gland were observed, but all these being of transitory nature nobody became incapable of their daily fatigue duty.

Experimental:

(1) Results of vaccination performed at Batavia.

Infected monkey-brain of 3rd generation was used as vaccine, in an amount of 2cc. This being done in April 21, 1944, the state of the spontaneous infection was observed to find out no case of infection among 37 persons of A-group, during 3 months after the vaccination. And one typical dengue was found from 5 non-vaccinated persons.

Next, among B-group (24 vaccinants) one contracted dengue at Surabaya just 2 weeks after vaccination. This, however, may be assumed as the contraction during the immunity has not yet fully developed.

(2) Results of vaccination made at Timahi near Bandoug.

Sixteen volunteers from a Japanese troop were vaccinated with the vaccine, prepared by the guinea pig-brain, infected with Fukuda (or Okajima) strain.

Nine volunteers were vaccinated with living virus-preparation; 7 volunteers were with formalized vaccine.

First dose (10/IV, 1944) 2 or 3cc, 2nd dose (15/IV, 1944) 3cc, and after 38 days (18/V, 1944), the immunity test was performed using an active virus taken from a patient (Furukawa). Titre of the patient serum being found as 10^{-5} by the intradermal injection, its 10^{-1}cc was injected to the 16 vaccinated volunteers, but all with only one exceptional case, who was vaccinated with the living vaccine, contracted typical dengue.

(3) Results of vaccination of A-group of Bandong.

Vaccine prepared from guinea pig-brain (Okajima-strain) was used in this case.

Ordinary vaccine was used in 1cc at 1st and 2cc at 2nd injections. Formalized vaccine was used in 2cc at Ist and 3cc at 2nd dose.

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After 42 days, 10^{-2}cc of the same patient serum (Furukawa) was injected to the above vaccinants. Two volunteers were inoculated with the same test-virus at the same time.

As the outcome of this susceptibility test, any protection was difficult to detect among persons, vaccinated with the formalized vaccine.

And, of the 3 persons, who have been vacinated with living-vaccine, one was found as completely refractory to dengue and the remaining 2 contracted dengue, but so mild as to be confined to bed only for 1.5 and 3 days, respectively.

Two freshly infected volunteers to serve as control and 3 previously vaccinated with formalized vaccine, altogether contracted typical dengue and were confined to their bed for 6 to 10 days.

Preeminence of living-vaccine was thus demonstrated.

(4) Results of B-group of Bandong.

Twenty volunteers were vaccinated. The vaccine was prepared by means of the monkey brain (5th generation), infected with Fukuda-strain.

First dose in 2cc (4/V, 1944) 2nd dose in 3cc (10/V, 1944). And on May 31, that is, 27 days after the first vaccination, 10 vaccinants tested on their resistance to dengue; 3 were injected with 10^{-2}cc and the remaining 7 injected with 10^{-3}cc respectively, of the same test virus as used in the preceding experiments. As the result, among 3, inoculated with 10^{-2}cc, one, severely and 2, slightly contracted dengue and among 7, which were infected with 10^{-3}cc of test-virus, only one contracted dengue, mildly.

Briefly:

1. Protection against dengue is attained by means of the attenuated mouse-virus.
2. More than a month seems to be necessary for the immunity produced by the vaccination to reach its maximum.
3. It was confirmed that we can protect against the infection of about 100 times of the minimal infecting dose of a active dengue-virus.
4. Presumably protection against dengue by means of the formalized vaccine, cannot be expected.

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EXPERIMENTAL STUDY ON MEASLES VIRUS

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In a previous communication, Yaoi and Arakawa (1) have described on inoculation experiment of measles virus upon mice by intracerebral injection.

Recently, the author has succeeded in the fixation of the same virus, by using the blood of a patient(2).

The virus causes the same symptoms in mice, and became "fixed" after 4 passages through mice: One of the 10 first generation mice was killed under doubtful symptoms and obtained the 2nd generation in which, 3 of 10 mice became ill within 3 to 14 days and died in 10 to 30 days.

In the 3rd generation, 5 of 5 animals fell ill on the 3rd to 11th days. In all the succeeding generations all mice inoculated contracted the disease and died on about 5th day, thus, the fixed virus was obtained.

Infected mice are very irritable, on being touched they would cry "Chi-Chi" and lift their fore legs.

They assume an attitude as if to stand erect elevating half the body. Some times they bite experimenter's finger. Soon after, the mouse stretches forth the legs and tremble, and after 2 or 3 hours at the most, bends its body backwards like a bow and dies in rigidity.

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Conjunctivitis, depilation, and diarrhea are found rather often. In very rare instances, papules or vesicles were produced on some part of the tail. Swelling was seen also at the middle part of tail.

In one case, swelling produced about the joint of the left fore leg and tail changed to haemorrhagic necrosis on the following day. From time to time, the depilation with fine yellowish bran-like desquamation is seen.

In another case, yellowish crust appeared symmetrically on the front aspect of the knee, followed by exfoliation and the spot remained unhaired and shiny. Furthermore, necrotic patches on the tip of tongue, poppy seed in size, and purpuric coloration of the tip of tongue were rather frequently seen.

At autopsy, pulmonary haemorrhage was seen in almost all cases.

Gray-coloration of the liver in varying grades was also very frequent.

Pathologic changes in spleen were not distinct. Swelling and haemorrhage of the kidney was seen in some cases.

Although some encephalo-meningeal symptoms such as chronic convulsion associated with opisthotonus and stretching of legs and vigorous trembling of body, etc., were characteristically observed in the beginning, following the passage, through many generations encephalomyelitic symptoms, such as paralysis of the legs and death in comatose state, became very marked. Dancing-movement was seen from time to time. The heightened irritabillity, seen in the early stage of the disease, however, remained unaltered.

Any inoculation method, intracerebral, intraocular, subcutaneous, intraperitoneal, intravenous, or intranasal, caused the mice to be fatally infected.

In addition to the mouse, the guinea pig and albino rat can also be incoulated with fatal outcome by intracerebral injection.

In the inoculated monkey, leucopenia and some rise in temperature is observed by the 5th or 7th day.

The virus passes readily through the gradocol membrane of 290 mμ and 175 mμ. Moreover, it passes thorugh the Seitz-EK filter without marked drop in virulence.

The virus theñ is evidently filterable and its size is considered by the author to be nearly the same in size or probably smaller than the virus of Japanese epidemic encephalitis (end-point 60 mμ) as estimated by Yaoi and others [3].

The virus is uninfluenced by glycerine and phenol. It is affected by X-rays and is inactivated when the dose is increased.

Active and passive immunization can be established by this virus. The mice, immunized with the virus material obtained from infected mouse brain, regardless of whether the virus is living or attenuated by the X-ray, are capable of withstanding the infection. It is especially noteworthy that the virus, attenuated by the X-ray, exhibits very potent antigenic properties.

The virus is neutralized by the adult human serum.

The mice on a diet, deficient in vitamin A and C, are more susceptible to the virus.

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INVESTIGATIONS IMMUNOLOGIQUES DU VIRUS DE LA FIÈVRE DE RIFT VALLEY

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Le pouvoir vaccinant du virus inactivé

Il est généralement admis que le virus de la fièvre de Rift Valley peut être inactivé par le formol tout en gardant sa fonction immunogène.[1] Le pouvoir vaccinant d'une émulsion du virus formolé, est-il conditionné par un petit nombre du virus vivant qui a échappé de l'action du formol? Chez la Souris, espèce très sensible a ce virus, injectée avec le virus formolé, on n'arrive à dèceler le virus actif ni dans le foie, ni dans le sang circulant, même en essayant des inoculations successives. Notons d'ailleurs que les souris ayant résisté à l'inoculation de virus à des doses très voisines à la dose mortelle ne se montrent jamais réfractaires à l'inoculation d'èpreuve.

Il est connu que le virus de la fièvre de Rift Valley fait produire les anticorps chez le Lapin et le Cobaye, espèces chez lesquels le virus ne se multiplie pas.[2]

Ces faits observés ne légitiment pas la notion classique: "sans infection, pas d'immunité". Le pouvoir vaccinant du virus de Rift Valley ne dépend pas de son pouvoir multipliant.

Les propriétés immunogènes du virus tué, diffèrent-ils de ceux du virus vivant? En comparant les doses de virus nécessaires pour faire apparaitre les anticorps chez le cobaye, nous avons constaté que la dose minime immunisante du virus vivant est de 20 à 200 fois la dose mortelle pour la souris alors que celle du virus formolé est de 2.000 à 20,000 fois la dose mortelle. Nous avons vu ainsi que le pouvoir antigène du virus est affaibli considérablement par action du formol.

En ce qui concerne le moment où les anticorps apparaient dans le sang chez l'animal vacciné, nous avons constaté que le virus tué, en dehors de toute attente, immunise plus vite que le virus vivant. Voici un exemple des expériences. Un matériel virulent (le sérum des souris infectées) est divisé en deux parties. Une moitié est ajoutée avec le formol à 0.9 pour cent et laissée à la température de laboratoire pendant trois heures, l'autre moitié ne reçoit aucun traitement. 0 cc 3 de chacun est injecté aux cobayes par

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voie souscutanée. Les animaux ainsi vaccinés sont saignés au bout des intervalles variés, leur sérums sont attestés contre une dose fixe de virus. Les résultats sont démontrés dans le tableau 1.

Tableau 1.

Immunogène		Pouvoir protégeant de sérum au deuzième jour	au troisième jour	au quatrième jour	au cinquième jour	Sérum du quatorzième jour protège à la dilution de
Virus vivant		+ 2	+ 2	+ 3	+ 3	1 : 100
		+ 2	+ 2	+ 3	+ 4	
		+ 3	+ 2	+ 3	+ 6	
Virus formolé		+ 2	+ 3	+ 4	+ 5	1 : 10
		+ 2	+ 3	S	S	
		+ 2*	+ 4	S	S	

(+ 2 désigne que la souris est morte au deuzième jour.)
(S désigne les souris ayant survécu.)

En titrant les sérums du quatorzième jour des cobayes vaccinés, nous avons assuré que le virus vivant immunise plus fortement que la même quantité du virus tué. Et pourtant, les anticorps sont décelables déjà au quatrième jour chez le cobaye vacciné avec le virus tué, tandis qu' ils n'apparaient pas encore au cinquième jour chez l'animal injecté avec le virus vivant.

Nous ignorons quel est le mécanisme par lequel le virus formolé fait apparaître les anticorps dans le sang plus vite que le virus non traité.

Investigation quantitative de la séroprotection

C'est connu que la neutralisation du virus par l'immunsérum n'obéit pas à la loi des proportions déterminées.[3] Nous avons cherché à savoir, au point de vue quantitative, quels sont les facteurs déterminant le succès ou l'échec de la séroprotection.

Le virus a été dilué décimalement sur une echelle aussi grande que possible. L'immunsérum a été dilué à 1:10, 1:100 et à 1:1000. Les dilutions de virus et celles de serum ont été mélangées à parties égales en toutes les combinaisons possibles. Les mélanges sont injectés aux souris par voie intraveineuse. Les résultats sont consignés au tableau 2.

Tableau 2.

Dilutions de sérum	Dilutions de virus 10	10^2	10^3	10^4	10^5	10^6	10^7
10	S	S	S	S	S	S	S
100	+	+	+	+	+	+	S
1000	+	+	+	+	+	+	+

Ce n'est pas la proportion relative entre le virus et les anticorps qui décide le succès ou l'échec de la protection par l'immunsérum, c'est uniquement la concentration dans laquelle l'immunsérum est présent dans l'organisme de la souris. Au-dessous d'une certaine

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concentration, les anticorps ne protègent plus l'animal même contre une faible quantité de virus. Au-dessus de cette limite de concentration, par contre, l'immunsérum protège la souris contre million fois la dose mortelle de virus.

Quelque abondant que soit le virus, l'immunsérum protège-t-il toujours l'espèce sensible quand il est présent à une concentration supérieur à ce seuil? Nous avons essayé les séroprotections contre des doses très élevées de virus. Le tableau 3 enregistre les résultats de ces essais.

Tableau 3.

Dilutions de sérum	Dilutions de virus: 1	2	5	10	10^2	10^3	10^4
1	S	S	S	S			
2	+	S	S	S			
5	+	+	S	S			
10	+	+	+	S	S	S	S
10^2	+	+	+	+	+	+	+

On voit ici que la protection obéit à la loi des proportions déterminées.

Influence de l'immunsérum sur le pouvoir vaccinant de virus

Contrairement à nos conceptions d'autrefois[1], le virus neutralisé par l'immunsérum peut, tout au moins dans certains cas, vacciner la souris[3].

Les souris ayant résisté à l'injection des mélanges de virus et d'immunsérum en proportions variées ont reçu une réinoculation d'épreuve au bout d'un intervalle de quatre semaines. Le tableau 4 démontre les résultats de l'épreuve.

Tableau 4.

Dilutions de l'immunsérum	Dilutions de virus: 10	10^2	10^3	10^4	10^5	10^6
1	S	+	+	+		
10	S	+	+	+	+	
10^2				+	+	+
10^3						+

On voit que le pouvoir vaccinant d'un mélange est déterminé par la quantité du virus contenu dans le mélange, mais non pas par la proportion relative entre le virus et les anticorps. Autrement dit, le degré de neutralisation ne décide pas si le mélange est capable ou pas de vacciner l'animal.

C'est ainsi que nous avons constaté que les anticorps peuvent empêcher l'infection tout en respectant le pouvoir vaccinant de virus.

Absorption spécifique d'anticorps

L'immunsérum a été mis en contacte avec un excès de virus à 37° C ou à la température de laboratoire. Au bout de temps variés (quatre heures, un jour, deux jours, trois jours et quatre jours), les mélanges ont été chauffés à 65° C pendant une heure afin de détruire la propriété antigène du virus y contenu. En titrant le pouvoir protecteur des

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mélanges ainsi chauffés, nous avons trouvé que la quantité d'anticorps n'était point diminuée.

Au contraire, quand nous avons injecté les mélanges à la souris au lieu de les incuber in vitro, nous avons constaté une diminution remarquable d'anticorps (voir le tableau 5).

Tableau 5.

		Mélanges de virus et d'immunsérum				Immunsérum seul			
Intervalles en jours entre l'injection et la saignée		1	2	3	4	1	2	3	4
Dilutions des sérums à titrer	1:1	+	+	+	+	S	S	S	S
	1:2	+	+	+	+	+	S	S	S
	1:5	+	+	+	+	+	+	+	+

L'absorption d'anticorps peut se faire non seulement chez la souris, mais aussi chez le cobaye, espèce réfractaire au virus de Rift Valley. On arrive à absorber les anticorps avec un virus tué aussi bien qu'avec un virus vivant, mais non pas avec un virus dépourvu de pouvoir immunisant.

Résumés

1° Le virus de la fièvre de Rift Valley inactivé par action du formol fait apparaître les anticorps chez le Cobaye plus vite que le virus actif.

2° Pour la séroprotection, il existe une certaine concentration en dessous de laquelle l'immunsérum ne protège plus la souris même contre une petite quantité du virus. Au-dessus de cette limite, l'immunsérum protège contre million fois la dose mortelle de virus.

3° L'immunsérum peut enlever le pouvoir multipliant de virus tout en respectant son pouvoir vaccinant.

4° Dans la condition de nos expériences, l'absorption spécifique d'anticorps peut se faire in vivo, mais non pas in vitro. Elle se fait au moyen du virus tué aussi bien qu'au moyen du virus vivant.

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THE ACTION OF IMMUNE SERUM ON THE JAPANESE ENCEPHALITIS VIRUS CULTIVATED *in vitro**

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Introductory

There seems to be a consensus of opinion among workers who studied the activity of

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antiviral serum in tissue culture, that a virus once entered a cell cannot be influenced by the detrimental effect of antibodies.

Andrewes,[1] in 1929, carried out a detailed investigation on the effect of immune serum upon virus III in a modified Maitland medium. He observed that if immune serum was added to normal rabbit testis before the addition of virus no inclusion bodies formed, nor was the culture infective for rabbits. On the other hand, if the virus was allowed to remain in contact with tissue for 10 to 60 minutes at 37°C before the addition of immune serum, multiplication of virus readily occurred, despite the presence of abundant antibodies in the fluid bathing the cell.

In the same year, Rivers, Haagen and Muckenfuss[2], studied the problem with vaccinia virus and rabbit cornea tissue cultivated by means of the classical cover-slip method. Pieces of cornea tissue, either infected *in vivo* or *in vitro* before the cultures were set up and placed in immune plasma, showed typical vaccinal lesions with Guarnieri bodies after 24 to 48 hours' incubation at 37°C. Moreover, the tissues were found to be virulent upon animal inoculation.

Thereafter, similar results were obtained by a number of research workers with a variety of viruses; *e.g.*, herpes virus (Andrewes[3]), vaccinia virus (Stoel[4], Vieuchange and Stamatin[5], Miyazawa[6]), yellow fever virus (Haagen[7]), fowl plague virus (Hallauer[8]) and infectious ectromelia virus (Downie and McGaughey[ ]).

On the basis of these results the conclusion has been generally drawn that virus-neutralizing antibodies have little, if any, effect upon the virus already penetrated into the tissue. To quote from Andrewes'[1] explicit description: ".....it seems possible that the immune serum acts by preventing the virus from entering the cell, and that if it has failed to achieve this purpose, it is powerless to do anything else."

There are, however, a few workers who do not agree with the seemingly established opinion as described above.

Magill and Francis[10] (1937) found that a ferret immune serum added into the culture flask 60 minutes after infection, inactivated the epidemic influenza virus in the tissue completely, whereas a less potent rabbit serum was unable to prevent the multiplication of the virus that had already been in progress.

Some part of Umetani's[11] experiments (1938) with vaccinia virus yielded results similar to those of the authors just mentioned above.

The following experiments were undertaken as part of my systematic investigations[12, 13] on the *in vitro* cultivation of the Japanese encephalitis virus, the results of which seem to be contradictory to the current opinion concering the problem at issue as advocated by Andrewes, Rivers and others.

MATERIALS

Virus: Strain *Kalinina*, isolated by ourselves in 1935 and has been propagated exclusively in mice, was employed throughout these experiments.

Immune Serum: The serum of a horse, which had received subcutaneous injections of 10 per cent mouse brain virus twice a week, in doses increasing from 5 to 120 cc

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(total amount injected: approximately 2,250 cc) over a period of 5 months, was tested for viricidal properties, and stored in the ice-chest without preservatives.

The antibody content of the immune serum will be described under a separate heading.

Normal serum: A normal horse serum which had been sent from Hokkaido, was found to have no virus-neutralizing antibodies, and was used in the control experiments. As stated elsewhere, it is the common experience of workers in this country, that the majority of the so-called "normal" sera (including those of human being, rabbits, guinea pigs, horses, etc.) exhibit more or less marked neutralizing properties against the Japanese encephalitis virus, whereas sera collected in Hokkaido, where frank cases of encephalitis are known to be rare, are almost invariably free from antibodies.

The Antibody Content of the Immune Horse Serum:

Tests for the viricidal properties of the serum (S No. 4) were performed as follows:

A 10 per cent suspension of a mouse brain virus was centrifuged for 30 minutes at 3,000 r.p.m. To each of the decimal dilutions of the supernatant fluid were then separately mixed equal parts of various dilutions of immune serum ranging from 1:10 to 1:10,000. Both virus and serum were diluted in meat infusion broth of *p*H 7.5.

Table 1

The Antibody Content of the Immune Horse Serum (S No. 4)

Virus \ Serum	1:1	1:10	1:100	1:1,000	1:10,000	Control
10^{-3}	10, S, S	S, S, S	9, 11, S	8, 9, S	6, 7, 7	6, 6, 7
10^{-4}	S, S, S	S, S, S	8, 10, S	7, S, S	7, 7, 8	6, 7, 8
10^{-5}	S, S, S	S, S, S	S, S, S	S, S, S	10, S, S	6, 7, 7
10^{-6}	△, S, S	S, S, S	S, S, S	S, S, S	S, S, S	7, 8, S
10^{-7}	S, S, S	S, S, S	S, S, S	S, S, S	S, S, S	△, 8, 9
10^{-8}			S, S, S	S, S, S	△, S, S	△, 8, 9

S .....Survived for 14 days.
8, 9 etc.....Mouse died after 8, 9, etc. days.
△.....Mouse died of accidents or intercurrent infections.

The mixtures were agitated thoroughly, then let stand for about one hour at room temperature, following which 0.03cc of each mixture was inoculated into each of three mice weighing 8 to 10 g. The mice were observed for 14 days.

The results are shown in Table 1.

Although serum diluted as highly as 1 in 10,000 showed a remarkable protection (more than 100 times of the m.l.d.), further test with a dilution of 1 in 100,000 revealed no protection whatever.

Experiment 1

The cultivation of the virus was performed in quite the same manner as recorded in

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my previous communication (13).

Into each of four Carrel's flasks, type D_5, was put 2.4 cc of Tyrode's solution *p*H 7.6, one drop (approximately 0.05 g. wet weight) of minced brain tissue of a chick embryo 9 to 11 days old, and 0.3 cc of a 10^{-6} dilution of mouse brain virus.

Cultures were then incubated at 35°C for 3 hours.

To each of the first two flasks was added 0.3 cc of immune horse serum. Accordingly, the actual concentration of the immune serum was 1 in 10 (*cf.* Table 1). All four were again incubated at 35°C.

After 4 days' incubation, the tissues of the first two flasks were separated by centrifugation, each of them was washed three times in nutrient broth* to free from the antibodies and was suspended in 0.3–0.4 cc of broth, which roughly approximated a 10 per cent suspension. (The sediment suspended in a small amount of broth can easily be sucked into a syringes with a 1/4 mm needle without grinding in a mortar.)

Each suspension was tested for virus by the intracerebral inoculation into four mice, and was found to be entirely negative.

Of the two control flasks, one was contaminated: the virus content of the other was determined quantitatively after 4 days' incubation. The infective titer of the tissue for mice was 10^{-5}, while that of the culture fluid was 10^{-}; results entirely in accordance with those described in my previous communications[12].

Experiment 2

Essentially the same results were obtained with cultures in a medium consisting of whole embryonic tissue (instead of embryonic brain tissue) and Tyrode's solution. No virus was found in a culture flask which received immune serum 3 hours after incubation.

In addition, 10 pieces of tissue from each culture flask, which had been in cubanited an immune serum-Tyrode medium for 3 days, were transplanted separately into plasma-clot medium and further cultivated at 37°C. Abundant growth of fibroblasts was observed after one or two days' incubation.

The facts indicate quite clearly that the absence of virus in the tissue of an immune serum-Tyrode medium is due to the direct action of the viricidal antibodies upon viruses; the possibility, even if quite improbable, of a noxious effect of the immune horse serum (containing not only antiviral, but also "anti-mouse brain" antibodies) upon the tissues, which makes them unsuitable for the virus to grow, is thus eliminated.

(As the embryonic *brain* tissue, with which the interference of the "anti-mouse *brain* antibodies" is more likely to occur, was difficult to test for its viability, a rather round-about experiment as described in this section was planned.)

Experiment 3

In the previous communication[13] on the cultivation of the Japanese encephalitis virus, I reported that the maximum titer of a culture was attained by the 4th or 5th day. With

* Each of the last wash-fluid was found to possess no viricidal action at all.

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these facts in mind, the above experiments for the detection of the virus were performed after 4 day's incubation at 35°C.

However, it may be argued that in the presence of immune sera the growth of the virus would possibly be retarded, so that tests for the virus at the 5th day of incubation might be regarded as rather premature.

The following experiments were planned in order to settle the question:

Ten culture flasks were prepared in quite the same manner as described in Exp. 1. After 3 hours' incubation at 37°C, into each of the six flasks was added 0.3 cc of immune serum, the remainder each received 0.3 cc of normal serum. All of them were again incubated at 35°C.

Three days later, the tissue of one of the six flasks was tested for virus exactly following the technique described above. Each of the remaining five were also tested after 5, 7, 10, 14 and 21 days' incubation respectively.*

The results of these tests together with those of the controls are shown in Table 2.

Table 2

Test for Virus after Various Period of Incubation

Duration of Incubation	Culture in imm. Serum + Tyrode	Controls: Norm. Serum + Tyrode
3 days	S, S, S, S	6, 6, 6, 7
5 days	S, S, S, S	
7 days	△, S, S, S	6, 6, 7, 7
10 days	△, S, S, S	
14 days	S, S, S, S	7, 7, 7, 8
21 days	S, S, S, S	

From the results of these experiments it would seem quite clear that annihilation, instead of mere retardation of multiplication, of the virus actually occurred *in vitro*.

Experiment 4

The preceding experiments indicated that no virus could be demonstrated in a culture flask into which was added a potent immune serum 3 hours after the cultivation was initiated.

That actual infection of the tissue with virus occurs within a short period of contact, say 30 minutes or less, is the experience common to most workers in the field of virus cultivation. Therefore, the results of the above experiments may be regarded as evidence that even a virus which has entered a cell can be influenced by antiviral antibodies.

Further experiments were planned in order to obtain a more precise information concerning the activities of the immune serum against the virus in tissue culture.

Cultures were put up as described in Exp. 2 and 3.

* For the details of the rise and fall of the encephalitis virus *in vitro*, readers are referred to my previous communication (13)

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After 24, 48 and 72 hours' incubation, flasks, two at each time, received immune serum, and again incubated at 35°C.

On the 6th day from the commencement of cultivation, the content of each culture flask was centrifuged and both the fluid and the tissue (after complete washing) were tested separately for the presence of virus by the intracerebral inoculation into mice.

The results are shown in Table 3.

Table 3 (a)

Tests for Virus in the Flasks into which Immune Serum was added after Prolonged Contact with the Virus.

Imm Serum added after	1 day		2 days		3 days	
Flask No	3	4	5	6	7	8
Fluid	S, S, S, S	S, S, S, S	S, S, S, S	S, S, S, S	10, S, S, S	9, S, S, S
Tissue	△, S, S, S	S, S, S, S	S, S, S, S	S, S, S, S	S, S, S, S	7, 7, 7, 7

Table 3 (b)

Control Flask with Normal Sera.

	Normal Serum after 1 Day	
Flask No.	1	2
Fluid	5, 5, 6, 7	5, 6, 6, 6
Tissue	6, 6, 7, 7	6, 6, 6, 6

As given in Table 3, immune horse serum added into the flask after 24 hours' incubation completely neutralized the virus; in the tissue of one of the flasks, which received the serum after 48 hours, virus was demonstrated, while that of the other was found to be sterile; the immune serum, which was added into the culture medium after 72 hours' incubation, was unable to inactivate the virus in the tissue cells. Moreover, it should be noted that in the last two flasks, virus was also demonstrated in the fluid medium, showing that the virus content of the fluid at the 4th day of cultivation was slightly beyond the limit of the viricidal activities of the immune serum employed (*cf.* Table 1, also Kawakita [13]).

Repeated experiments yielded results similar to those described above.

These results seem to be dependent upon the quantitative relationship between the potency of the immune serum and the virus content of the tissue at the time of their meeting.

If we compare the above findings with the informations given in my previous communication[13], it may probably be estimated that, under the specified conditions of our experiments, a tissue whose infective titer is below 10^{-3} (or maybe 10^{-}) can always be rendered inative by the viricidal activities of our potent immune horse serum.

Experiment 5

In the following experiments tissues infected *in situ* were employed for the cultivation

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in an immune serum-Tyrode medium.

Developing hen's egg, each of them 9 days old, were inoculated into the chorioallantois with 10 per cent supension of the mouse brain virus, and were incubated at 37°C.

Twenty-four hours later, the embryo was removed aseptically; the brain was enucleated, carefully freed from meninges, and divided into halves.

The one hemisphere was emulsified and tested for the infective titer by the customary manner, while the other was washed with Tyrode's solution and minced with scissors.

Into a Carrel's flask was put one drop of the above-mentioned infected brain tissue pulp, 2.7 cc of Tyrode's solution and 0.3cc of immune resp. normal serum. Flasks were then incubated at 37°C.

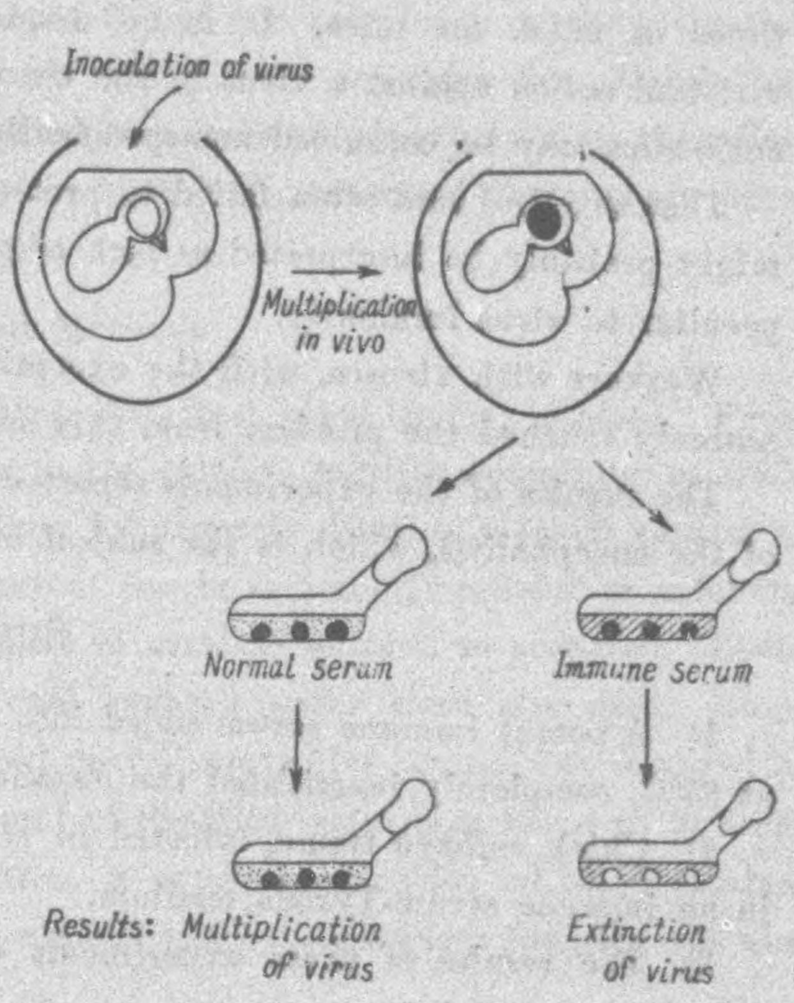
After 5 days' incubation, the virus content of the flasks was determined by the intracerebral inoculation of mice.

The results of these experiments are shown in Table 4 (see also Text-Fig. 1).

Table 4

Experiments with Tissue infected *in situ*

Egg No.		1	2	3
Titer of brain tissue at the time of enucleation		10^{-4}	10^{-2}	10^{-4}
After Cultivation in Serum (imm resp norm.) Tyrode +	Fluid	6 6 6 6	△ S S S	S S S S
	Tissue	6 6 6 7	S S S S	9 S S S
		Normal Serum	Immune Serum	



Text-Fig. 1. Illustration of the Experiment 5.

Interpretations of the results of these experiments are omitted.

DISCUSSION

As stated before, it seems to be a wellnigh established opinion among research workers in the field of viruses, that a virus once entered a cell is perfectly protected against the activities of immune serum.

However, in the light of the results of the above experiments, the opinion seems to have gone too far. As a matter of fact, there seems to be no doubt that a virus in a cell offers a marked resistance to the harmful effect of chemicals, antibodies, photodynamic action of dyes etc., as compared with the virus in a cell-free suspension.

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Under the conditions of the experiments described above, however, immune serum added into the culture flask after infection had actually occurred, was able to check the mutiplication of the virus in the tissue cells. In fact, the results of Exp. 3 indicated that after 24, sometimes even after 48 hours' incubation at 35°C, the virus in the tissue completely succumbed to the viricidal activities of the immune serum.

Whether the discrepancy between our findings and those of other workers may to some extent be due to the difference of the virus employed or not, is a problem which needs further elucidation. In our opinion, however, it seems to be more likely that the results of the experiments like these, depend upon the quantitative relationship between the two opposed forces, namely, the potency of the immune serum and the virus content of the tissue.

It cannot be overlooked that the immune horse serum employed in our experiments was extremely potent; a 1:10,000 dilution of the serum neutralized several hundred times of m.l.d. for mice. It is no wonder that such a potent serum can exhibit its viricidal action against a virus in the tissue cells, although a considerable amount of the antibodies may be consumed non-specifically.

That antiviral sera often failed to protect a tissue after the establishment of infection, might probably be interpreted as lack of potency of the serum employed, not as a feature peculiar to virus immunity.

Workers with viruses, with the exception of Magill and Fracis, seem to have hitherto scarcely touched the problem from this angle.

The results of the experiments reported here, suggest the possibility of serum therapy of the encephalitis, which is the subject of our next communication.

Summary

1) A potent immune serum added into a culture flask after 24 to 48 hours' incubation at 35°C, completely inactivated the Japanese encephalitis virus in the tissue cells.

2) Chick embryo tissue, infected *in situ*, was also rendered sterile after cultivation in an immune serum-Tyrode medium.

3) The results of these experiments suggest the possibility of serum therapy of the Japanese encephalitis.

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STUDIES ON THE ACTIVATION OF CATALASE ACTIVITY

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The catalase action, especially the question of its inhibition, has been the subject of much discussion by many previous workers, (1–6) but our knowledge on the activation of the action remained scanty, and, as yet, no indisputable activator for catalase has been established. In the present paper, based on an extensive experiments on the effect of inorganic salts upon the catalase activity, the author demonstrated that certain phosphates and carbonates act as powerful activators.

Material and Methods

Experiments were carried out with liver (rat) and blood (horse) catalase. From the nature of the experiments it is desirable to use catalase solution containing as little salt as possible. In the case of liver catalase, by properly diluting a small amount of liver extract with distilled water, the salt content can be sufficiently reduced to meet the experimental requirement, and the use was made of catalase solution so prepared. Liver extracts made salt-free by dialysis against cold running water were also used. Blood catalase solution prepared by extracting blood cake, from which serum was removed as completely as possible, with appropriate amount of distilled water was found to be sufficiently salt-poor for our purpose. In addition, a crude preparation of blood catalase obtained by removing hemoglobin with chloroform and by subsequent dialysis was also used.

The hydrogen peroxide solution used was a commercial porduct, but preliminary tests satisfied the author that it does not contain any impurity which would materially modify the effect of salts.

Catalase activity was measured mostly by gas-volumetric method with the aid of a Battelli-Stern apparatus, but all the more important results were confirmed using titration with potassium permanganate solution.

Experimental Results

The following 28 inorganic salts were used in the experiments:— $MnSO_4$, $MnHPO_4$, $MnCO_3$, $MnCl$, $BaSO_4$, $BaHPO_4$, $BaCO_3$, $BaCl_2$, $CaSO_4$, $CaHPO_4$, $CaCO_3$, $CaCl_2$, $MgSO_4$, $MgHPO_4$, $MgCl_2$, $NaSO_4$, Na_2HPO_4, $MgCO_3$, $NaCO_3$, NaCl, K_2SO_4, K_2HPO_4, K_2CO_3, KCl,

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NH_4SO_4, $(NH_4)_2HPO_4$, $(NH_4)_2CO_3$, NH_4Cl. In each case 50mg of salt was tested in 10cc of distilled water containing 0.1cc of catalase solution.

All the experiments yielded consistent results, regardless of the type of catalase solutions or of experimental methods: sulphates were essentially indifferent, chlorides tended to be inhibiting, while phosphates and carbonates generally produced activating effect.

The activating effect was especially marked in Na_2HPO_4, $(NH_4)_2HPO_4$, $BaCO_3$, $CaCO_3$, $MgCO_3$, $(NH_4)_2HPO_4$, etc., and the catalase activity was increased by several hundred per cent through the addition of one of these salts.

An experiment with dilute liver extract may be cited as a typical example in the following table, where, to facilitate comparison, all the figures (cc of oxygen) are recalculated in proportion, taking the figures for the control without the addition of salt as 100. The salts are conveniently designated by their cations and anions.

	SO_4''	PO_4''	CP_3''	Cl'
$Mn^{\cdot\cdot}$	71.6	116.9	390.5	39.6
$Ba^{\cdot\cdot}$	19.2	403.7	490.0	79.2
$Ca^{\cdot\cdot}$	100.0	350.9	567.6	58.1
$Mg^{\cdot\cdot}$	70.9	390.5	476.7	19.2
$Na^{\cdot}$	86.0	587.8	93.0	23.4
$K^{\cdot}$	81.5	215.1	242.1	41.5
$NH_4^{\cdot}$	73.8	560.0	555.3	19.2

It may be readily seen that the magnitude of catalase activity differs widely according to the kinds of salt added. Two phosphates and four carbonates, namely, Na_2HPO_4, $(NH_4)_2HPO_4$, $BaCO_3$, $CaCO_3$, $MgCO_3$, $(NH_4)_2CO_3$, are notable in being associated with very high activity, while all the chlorides, especially $MgCl_2$, NH_4Cl and $NaCl$, and also $BaSO_4$, may be pointed out as accompanying maredly reduced activity.

These divergent results cannot be attributed to different hydrogen ion concentrations. With the exception of $BaSO_4$, $NaCO_3$, and K_2CO_3, which showed strongly alkaline reaction of over pH 9.6, almost all the salts, when 0.05g of each was dissolved or suspended in 10cc of distilled water, gave reactions varying little around pH 5–6, there being no consistent difference between the "activating" and inhibiting groups of salts. For the group of six highly "activating" salts the reactions were: Na_2HPO_4, pH 8.0; $(NH_4)_2HPO_4$ pH 8.6; $BaCO_3$, pH 5.0; $MgCO_3$, pH 5.8; $(NH_4)_2CN_2$, pH 8.6. Neither can the solubility of the salts be significant in this connection, since, although $BaCO_3$, $CaCO_3$, and $MgCO_3$ are insoluble in water, equally "activating" Na_2HPO_4, NH_3HPO_4 and $(NH_4)_2CO_3$, are readily soluble.

The old contention of Michaelis and others (3) that in salt influence on catalase activity the anions play more deciding rôles than do cations seems to be well substantiated by our results, which also confirm the position of chlorides as outstanding inhibitors of catalase activity.

The point of departure from these previous studies is provided by our observations of

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the greatly increased catalase activity in the presence of certain phosphates and carbonates. Here it may be worth while to state that it was through the use in each test of a very small amount (0.1cc–0.2cc) of catalase extract that the "activating" effect of these salts was demonstrated. In dealing with such a crude material as the whole liver extract as we did, this effect would not have come to light had we used the extract in much larger amounts. The use of as much as 1cc of the extract introduces sufficient amount of salts for the maximum activity of catalase and superposition of additional salt produces no "activating" effect. This would mean, incidentally, that the amount of salt necessary for maximum catalase activity must be fairly small and that larger amounts of salt over and above this necessary minimum do not produce any additional effect.

The concentration of salt solutions necessary to maintain their optimum activating effect varied: 1/500 mol for Na_2HPO_4, 1/250 mol for $(NH_4)_2CO_3$, 1/60 mol for $(NH_4)_2HPO_4$, and 1/4 mol for $NaCo_3$ and K_2CO_3, higher concentration over these minima producing no corresponding increase in activation.

Mixtures of two activating salts produced intermediate effect, but exceptional cases were found in which the activating effect of a mixture far surpassed the sum of the effect of the two component salts, the mixture of Na_2CO_3 or K_2CO_3 and $MnHPO_4$ being the most striking examples of this kind.

Discussion aud Additional Experiments

As stated above certain phosphates and carbonates bring about an apparent activation of catalase activity, but before the results can be accepted as evidence of true activation, it is necessary to examine other possible causes which may account for the phenomenon.

It may first be recalled that Charmandarjan and Tiutjunikowa (7) expressed the view that K_2HPO_4, Na_2HPO_4, and $NaHCO_3$ activated malt catalase, based on the findings that catalase extracted with solutions of these salts was much more active than that made with distilled water. This view was contested by Zeile (6), who explained that the enzyme in distilled water may be in weakened condition, instead of the salts having activating action. The present author's experiments, however, conclusively showed that liver or blood catalase in distilled water extracts is just as potent as that in phosphate or carbonate solution extracts, inasmuch as they showed equal degree of activity when allowed to react with H_2O_2 in the medium of either salt solution or of distilled water.

Catalase solution was prepared by thoroughly extracting the same amount (1 g) of the same rat liver with the same amount (50cc) of distilled water and of Sörensen's phosphate buffer solution (pH 7), and it was found the potential catalase activity of the extracts were identical:—

0.2cc phosphate extract diluted with 10cc phosphate solution when tested with H_2O_2 yeielded 10.4cc oxygen in 5 minutes; the same diluted with 10cc distilled water, yielded 4.2cc oxygen.

0.2cc distilled water extract diluted with 10cc phosphate solution yielded 10.6cc oxygen; the same diluted with 10cc distilled water, 4.4cc oxygen.

The catalase in distilled water extract is by no means in weakened condition, inasmuch

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as it shows equal degree of activity to that in phosphate solution extract, when tested in the medium either of phosphate solution or of distilled water.

A unique feature of the catalase activity is the remarkable vulnerability of the enzyme to its substrate (H_2O_2), and if phosphates and carbonates protected catalase from destruction by H_2O_2, they can bring about the apparent activation of catalase. It was found, however, that the activating effect of the salts is shown most markedly in the first one minute of the reaction with H_2O_2, when the destruction of catalase must be the least, proving that the effect of the salts cannot be attributed to the protection of catalase from destruction.

0.2cc of fresh liver extract was diluted with 10cc of distilled water, either alone or with the addition of 0.05 g of Na_2HPO_4. 2cc of H_2O_2 solution was then added and the reaction was stopped with 10cc of 10% H_2SO_4 solution at the end of every 1 minute for 5 minutes. 10/N solution of $KMnO_4$ was used for the titration of the remaining H_2O_2. The amount of H_2O_2 solution (2cc) was so chosen that H_2O_2 became almost all consumed in 5 minutes by the amount of catalase extract used in the presence of Na_2HPO_4.

Using as the basis for calculation the titre (cc) for 2cc of the H_2O_2 solution, we obtained the amount of $KMnO_4$ solution corresponding to that of H_2O_2 consumed for every one minute period:—

	1st min.	2nd min.	3rd min.	4th min.	5th min	Total
With Na_2HPO_4	14.8	2.3	1.0	0.4	0.4	18.9
Distilled water alone	2.5	1.8	1.2	1.0	1.0	7.5

If the function of the salt is to protect catalase from destruction by H_2O_2 the effect of it should appear more strikingly toward the end of the reaction than at the beginning, but actually there is an overwhelmingly greater catalase activity with salt than without it only at the earliest period of the reaction, when the destruction of catalase must be the least.

Catalase solution may contain some inhibiting substance, which may be removed by the salt, so producing an apparently activating effect. This possiblity also failed to be substantiated, since the catalase solution treated with activating salts (using water-insoluble phosphates and carbonates) and subsequently freed from them by centrifugation, showed absolutely no increase in its activity.

We quote the following experiment as an example:

0.2cc of the enzyme extract was diluted with 10cc of distilled water and 0.05 g of $BaCO_3$ was added. After allowing to stand for 30 minutes with occasional shaking the mixture was centrifuged at a high speed and the catalase activity of the clear supernatant was compared with that of the control extract without the treatment with $BaCO_3$. The catalase activity, as measured with the aid of Battelli-Stern apparatus, was approximately the same for both $BaCO_3$ treated and untreated extracts:

Treated extract supernatant yielded 10.4cc oxygen; control extract, 9.6cc.

Similar experiments proved that the possible existence of inhibiting substance in, and

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Treated extract supernatant yielded 10.4cc oxygen; control extract, 9.6cc.

Similar experiments proved that the possible existence of inhibiting substance in, and

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removal of it by salt from the H_2O_2 solution, cannot account for the observed effects.

Catalase once exhausted by reaction with H_2O_2 does not regain its activity by the addition of phosphates or carbonates. Neither can the inactivation of catalase by heat, KCN, or hydroxylamine be prevented by any of these salts.

The activating effect of the salts on the decomposition of H_2O_2 is specific to the case of catalase activity, and the H_2O_2 decomposition by other substances such as PbO_2 or MnO_2 is not accelerated by these salts.

All these experimental results lead to the conclusion that certain phosphates and carbonates, especially Na_2HPO_4, $(NH_4)_2HPO_4$, $BaCO_3$, $CaCO_3$, NH_2CO_3, etc., activate in the true sense the catalase action of $2H_2O_2 \rightarrow 2H_2O + O_2$. These inorganic salts, then, constitute the **activators for catalase.** which are definitely established now for the first time.

Conclusion

In order to exhibit its maximum activity catalase requires the presence of certain inorganic salts, especially Na_2HPO_4, $(NH_4)_2HPO_4$, $BaCO_3$, $CaCO_3$, $MgCO_3$, and $(NH_4)_2CO_3$. Without the coexistence of one of these phosphates or carbonates the catalase activity may remain approximately 500 per cent lower.

These facts may prove of significance in determining the potency of purified samples of catalase. Other salts, so far as the present observations extend, seem to be more or less indifferent, while certain chlorides act definitely as inhibitors.

The author wishes to express her appreciation to Dr. Waro Nakahara, under whose guidance the present study was carried out.

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RESEARCHES ON THE CHEMOTHERAPY OF PROTOZOAL INFECTIONS

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VI. Chemotherapy of Trichomoniasis

Researches on the chemotherapy of protozoal infections have been carried out for several years in our laboratory for the purpose of discovering new methods of treating these discases.

I. Chemotherapy of Malaria

The therapeutic activity of numerous drugs on canary malaria has been determined by Rohr's method for plasmoquine test. With a view to pursuing the investigations, we sought the chemical collaborations of Dr. E. Ochiai and Dr. K. Tsuda of the Pharmaceutical Laboratory of University of Tokyo.

The effective drugs on canary malaria (*Plasmodium relictum*) were applied for human cases in order to confirm their activity against malaria.

(A) Sulfonamide Compounds

A total of 53 sulfonamide compounds were tested in this laboratory. Sulfadibromobenzene and sulfadichlorobenzene seemed to be appliable for human malaria as compared with the activity of quinine on canary malaria. Accordingly sulfadibromobenzene were applied for three tertian cases with desirable results. (Table 1, 2)

Table 1. Sulfonamide

Sulfanilamide
3,5-Dibromo-sulfaanilamide
Sulfaguanidin
p-Aminobenzolsulfonacetamide
Diseptal A
4 (4′-Amino-3′,5′-dibromobenzene-sulfonamide)-benzene-sulfon-dimethylamide
Diseptal B
Diseptal C
p-(p′-Aminophenyl)-benzene-sulfonamide
Sulfanilanilide
1-Sulfanilamido-3,5-dibromobenzene
1-Sulfanilamido-3,5-dichlorobenzene
Sulfapyridine
2-(4′-Amino-3′,5′-dibromobenzene-sulfonamido)-pyridine
2-Sulfanilamido-6-methylpyridine
3-Sulfanilamido-2, 6-lutidine
2-Sulfanilamido-5-chloropyridine
2-Sulfanilamido-5-bromopyridine

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2-Sulfanilamido-5-aminopyridine
2-(4′-Amino-3′, 5′-dibromobenzene-sulfonamido)-5-aminopyridine
Sulfathiazole
Sulfamethylthiazole
2-(4′-Amino-3′, 5′-dibromobenzene-sulfonamido)-4-methyl-5-bromothiazole
2-(4′-Amino-3′, 5′-dibromobenzene-sulfonamido)-5-bromothiazole
2-Sulfanilamido-4-methyl-5-carbothoxythiazole
4-Sulfanilamido-1-phenyl-2, 3-dimethyl-5-pyrazolone
2-Sulfanilamido-chinoline
3-Sulfanilamido-chinoline
2-Sulfanilamido-benzothiazole
2-Sulfanilamido-5-methoxy-benzothiazole
N^4-Dimethylsulfanilamide
Marfanilamide
Prontosil rubrum
3-Carboxy-4-aminoazobenzene-4′-sulfonamide
Rubiazol
Proseptasine
2-(N^4-Sulfanilylsulfanilamido)-4-methylthiazole
N′-Dimethyl-N^4-(N″sulfanilylsulfanil)-sulfanilamide
N′-Dimethyl-N^4-(N'^4-(N''^4-sulfanilyl-sulfanilyl)-sulfanilyl)-sulfanilamide
Phonocasil
2-(N^4(N'^4-Sulfanilyl-sulfanilyl)-sulfanilamido)-4-methyl-thiazole
2-Sulfanilamide-6-methoxy-benzothiazole
2-Sulfanilamido-6-ethoxy-benzothiazole
2-Sulfanilamido-5-bromo-benzothiazole
2-Sulfanilamido-6-chloro-benzothiazole
7-(4′-Sulfonamidophenylazo)-6-hydroxy-benzothiazole
7-(4′-Sulfonamidophenylazo)-2-methyl-6-hydroxy-benzothiazole
2-Sulfanilamido-pyrimidine
2-Sulfanilamido-4-methylpyrimidine
2-Sulfanilamido-4, 6-dimethylpyrimidine
1-Sulfanilamido-5-bromo-naphthalene
1-Sulfanilamido-4-bromo-naphthalene
1-Sulfanilamido-5, 8-dibromo-naphthalene

Table 2

	M.E.D.	M.T.D.	M.T.D.: M.E.D.
Quinine hydrochloride	1:800	1:200	1:4
Sulfadibromobenzene	1:200	1: 50	1:4
Sulfadichlorobenzene	1:400	1:200	1:2

M.E.D. . . . . Minimum effective dose
M.T.D. . . . . Maximum tolerated dose

(B) Benzothiazole and benzothiazolone (2) compounds

Sixteen benzothiazole and three benzothiazolone compounds were tested with negative results on canary malaria. (Table 3)

Table 3. Benzothiazole, benzothiazolone

2-Sulfanilamido-benzothiazole
2-Sulfanilamido-5-methoxy-benzothiazole
2-Sulfanilamido-6-methoxy-benzothiazole
Sulfanilamido-6-ethoxy-benzothiazole
Sulfanilamido-5-bromo-benzothiazole

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2-Sulfanilamido-6-chloro-benzothiazole
7-(4′-Sulfonamidophenylazo)-6-hydroxy-benzothiazole
7-(4′-Sulfonamidophenylazo)-2-methyl-6-hydroxy-benzothiazole
2-(β-Diethylaminoethylamino)-benzothiazole
2-(γ-Diethylaminopropylamino)-benzothiazole
2-(γ-Diethylaminopropyl-acetylamino)-benzothiazole
2-(γ-Diethylamino-β-oxy-propylamino)-benzothiazole
2-(γ-Diethylaminopropylamino)-5-methoxy-benzothiazole
2-(γ-Diethylaminopropylamino)-6-methoxy-benzothiazole
2-Amino-3 (γ-diethylaminopropyl)-benzothiazole
2-Amino-3 (γ-diethylaminopropyl)-6-chloro-benzothiazole
3-(β-Diethylaminoethyl)-benzothiazolone (2)
3-(β-Diethylaminopropyl)-benzothiazolone (2)
3-(γ-Diethylaminopropyl)-6-chloro-benzothiazolone (2)

(C) Quinazolone compounds

Nine quinazolone compounds were tested with negative results on canary malaria. (Table 4)

Table 4. Quinazolone (4)

6-Amino-quinazolone (4)
3-Methyl-6-amino-quinazolone (4)
3-Methyl-8-amino-quinazolone (4)
3-Methyl-6-chloro-8-Amino-quinazolone (4)
8-(β-Diethylaminoethylamino)-quinazolone (4)
3-Methyl-6-(β-diethylaminoethyl-amino)-quinazolone (4)
3-Methyl-7-(β-diethylaminoethyl-amino)-quinazolone (4)
3-Methyl-8-(β-diethylaminoethyl-amino)-quinazolone (4)
3-Methyl-6-chloro-8-(β-diethylaminoethyl-amino)-quinazolone (4)

(D) Quinazoline compounds

Fourteen quinazoline compounds were examined with some expectations in their activities on canary malaria. (Table 5)

Table 5. Quinazoline

4-(β-Diethylaminoethylamino)-quinazoline
4-(β-Diethylaminoethylamino)-8-nitro-quinazoline
4-(β-Diethylaminoethylamino)-6,8-dichloro-quinazoline
4-(γ-Diethylamino-α-methylpropylamino)-6,8-dichloro-quinazoline
4-(β-Diethylaminoethylamino)-6,8-dibromo-quinazoline
4-(γ-Diethylamino-α-methyl-propylamino)-6,8-dibromo-quinazoline
4-Methoxy-6-(β-diethylaminoethylamino)-quinazoline
4-Methoxy-8-(β-diethylaminoethylamino)-quinazoline
4-Methoxy-7-(β-diethylaminoethylamino)-quinazoline
4-Methoxy-7-(γ-diethylaminopropylamino)-quinazoline
4-(γ-Diethylamino-α-methylpropyl-amino)-6-methoxy-quinazoline
2-(γ-Diethylamino-propylamino)-quinazoline
4-Sulfanilamido-quinazoline
4-Sulfanilamido-6, 8-dibromo-quinazoline

(E) Benzotriazole compounds

Five benzotriazole compounds were tested with negative results on canary malaria. (Table 6)

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Table 6. Benzotriazole

5-Methoxybenzotriazine
1-(β-Diethylaminoethyl)-6-methoxy-benzotriazole
1-(γ-Diethylamino-α-methyl-propyl)-6-methoxy-benzotriazole
1-(δ-Diethylamino-α-methyl-butyl)-6-methoxy-benzotriazole
1-Methyl-3-(δ-diethylamino-α-methyl-butylamino)-4-amino-benzole

(F) Benzoquinoline compounds

Seven benzoquinoline compounds were tested with negative results on canary malaria. (Table 7)

Table 7. Benzoquinoline

7, 8-Benzoquinoline
2-Methyl-5-methoxy-6-acetylamino-7,8-benzoquinoline
2-Methyl-4-diethylaminoethylamino-5 6-benzoquinoline
2-Methyl-4-diethylaminoethylamino-7 8-benzoquinoline
2-Methyl-4-diethylaminoethylamino-6-methoxy-7 8-benzoquinoline
2-Methyl-4-diethylaminoisopentylamino-7,8-benzoquinoline
2-Methyl-4-diethylaminoisopentylamino-6-bromo-7,8-benzoquinoline

(G) Diphenylsulfone and approximate compounds

Sixteen compounds were tested with the results of undesirable action on canary malaria. Promin, as Coggeshall (1911) published, has a slight activity on canary malaria. (Table 8)

Table 8. Diphenylsulfone and approximate compounds

4, 4′-Diamino-diphenylsulfone
4, 4′-Diamino-3′, 5′-dibromo-diphenylsulfone
4-Amino-3′, 5′-dibromo-diphenylsulfone
4-Amino-4′-nitro-diphenylsulfone
4, 4′-Dinitro-diphenylsulfone
4-Nitro-diphenylsulfone
4-Nitro-3′, 5′-dibromo-diphenylsulfone
4-Nitro-4′-amino-3′, 5′-dibromo-diphenylsulfone
4, 4′-Diamidino-diphenylsulfone
4, 4′-Diamidino-diphenylsulfoxyde
4, 4′-Diamidino-stilbene
4, 4′-Diguanidino-diphenylsulfone
4, 4′-Diguanidino-diphenylsulfoxyde
4, 4′-Diguanidino-diphenylsulfide
Promin
4-Aminophenyl-3′-aminopyridyl-(6′)-sulfone-N, N′-didextrose sulfonate

(H) Quinine derivatives

Forty two quinine derivatives, collaborated by Dr. E. Ochiai, were examined and it was found that certain derivatives exhibited a malaricidal action on canary. (Table 9, 10)

Q 4 (6-Amino-dihydro-cinchonidin) was applied in five human tertian cases with satisfactory results.

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Table 9. Quinine deriva ives

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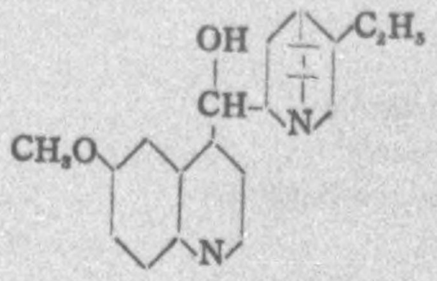

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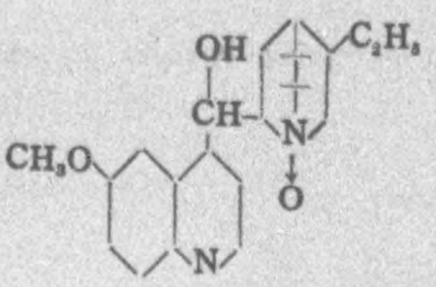

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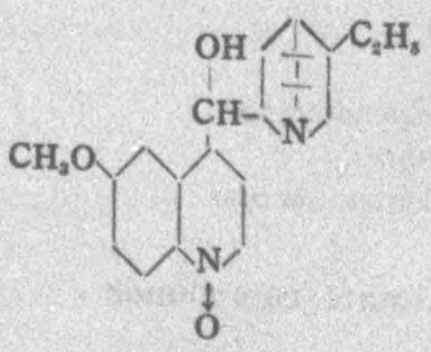

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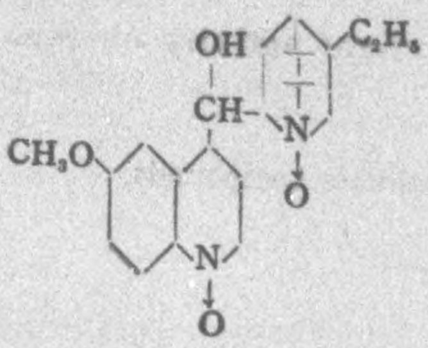

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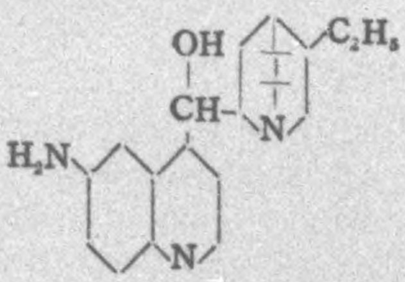

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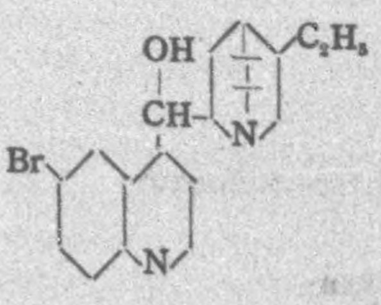

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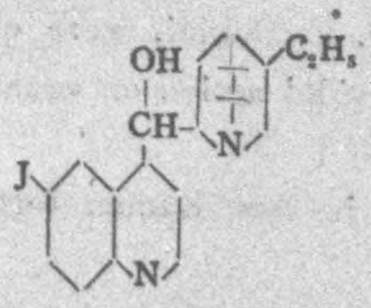

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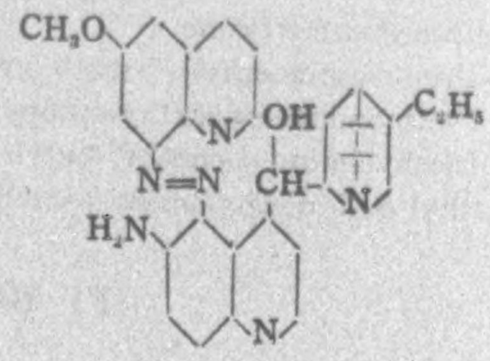

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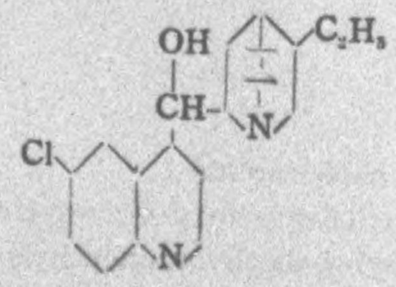

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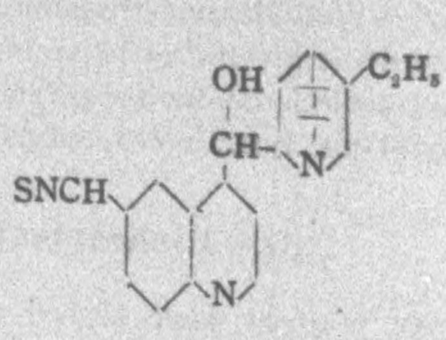

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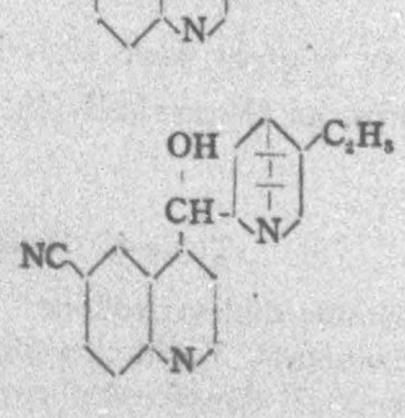

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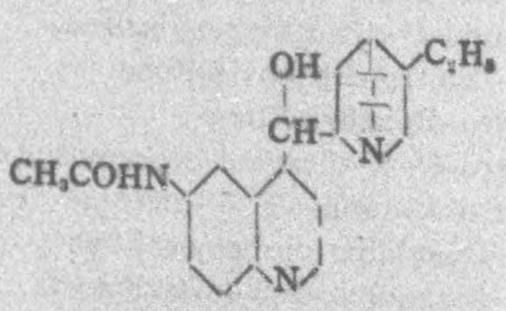

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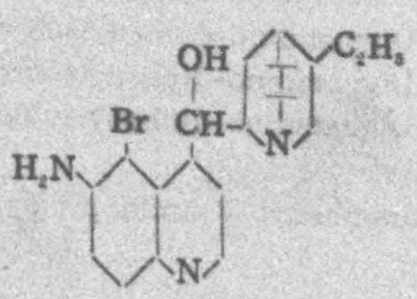

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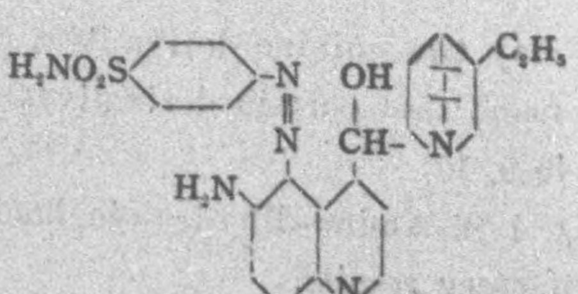

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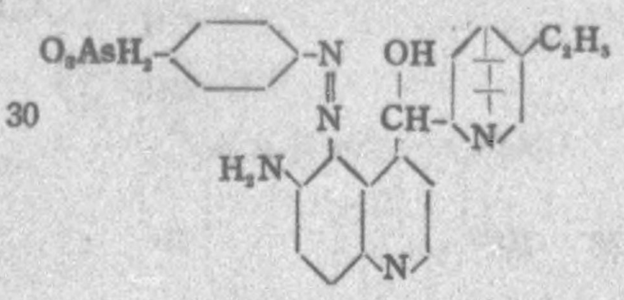

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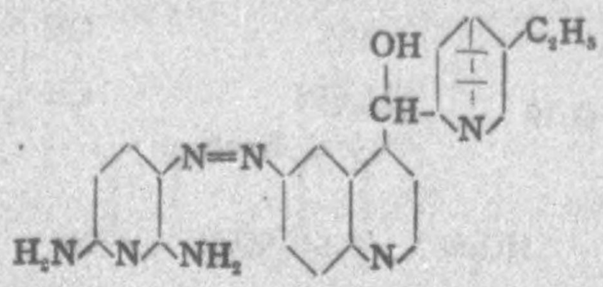

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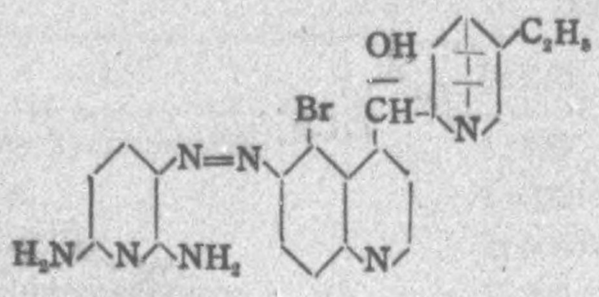

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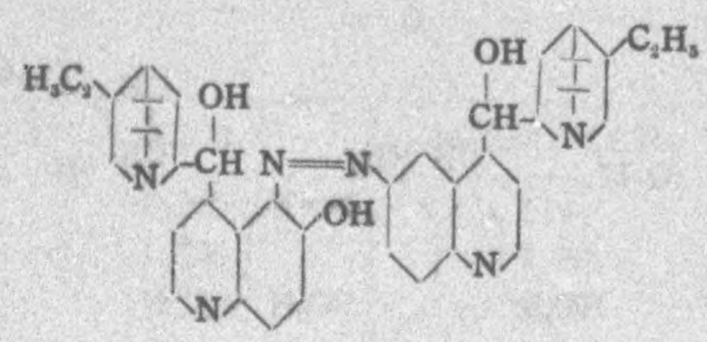

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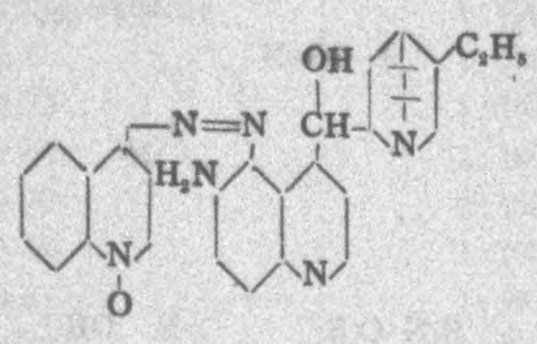

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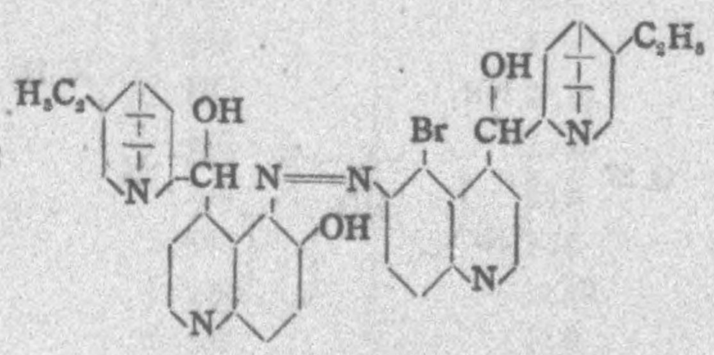

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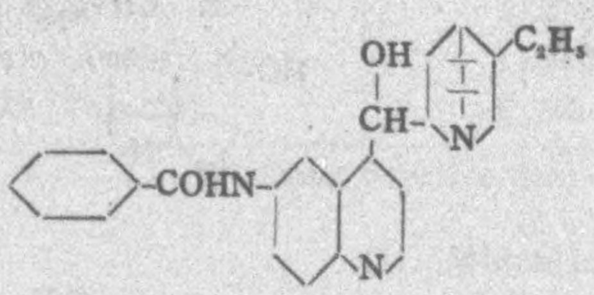

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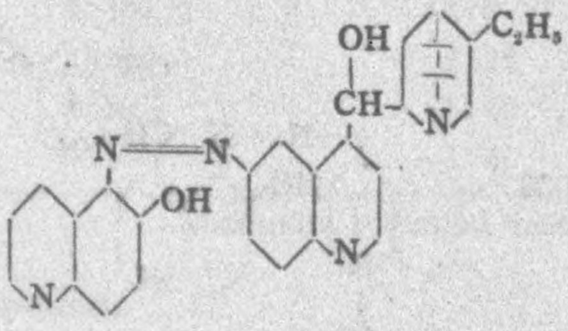

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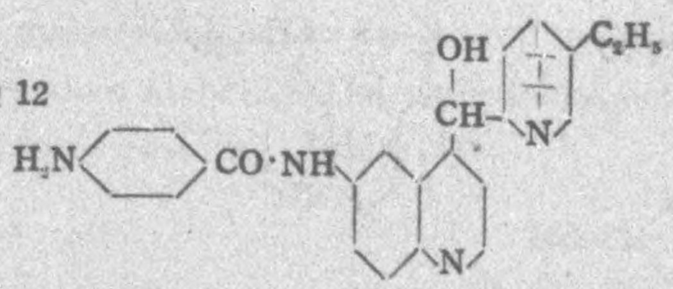

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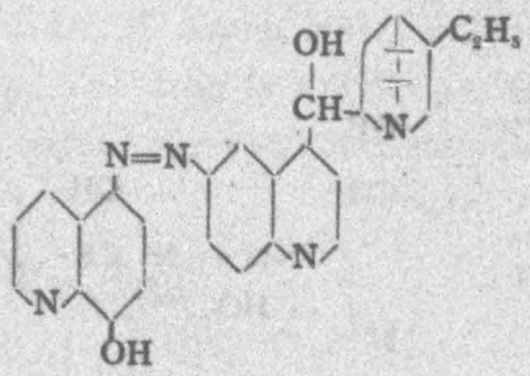

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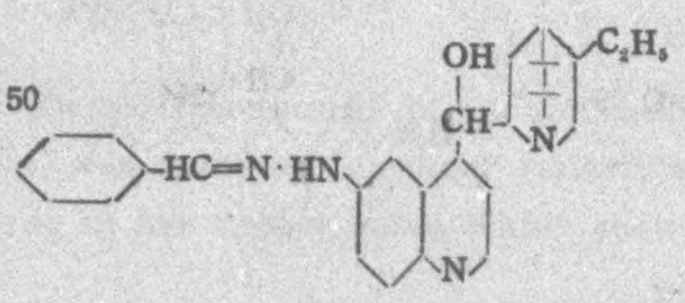

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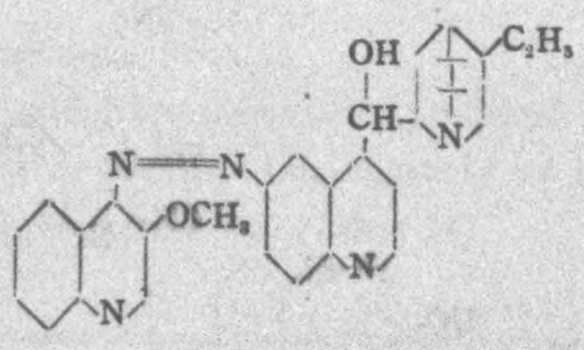

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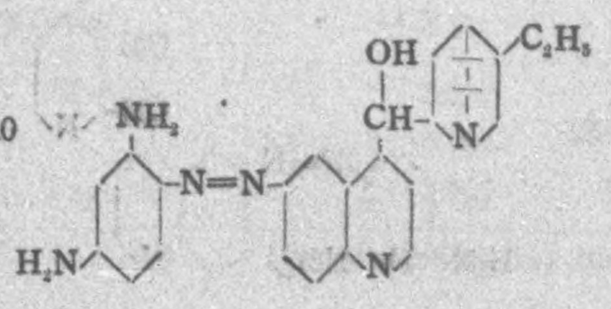

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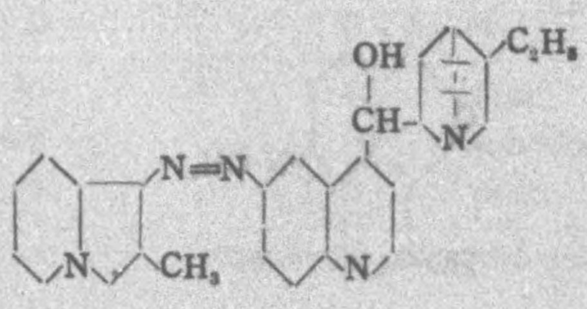

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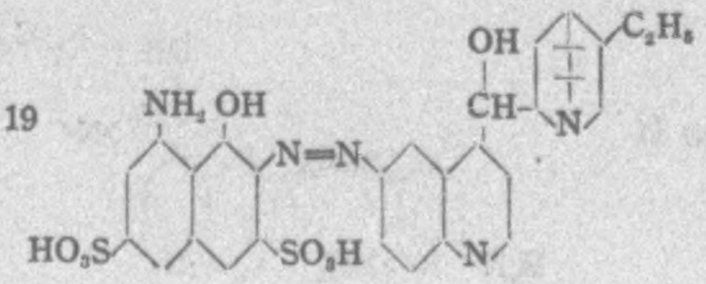

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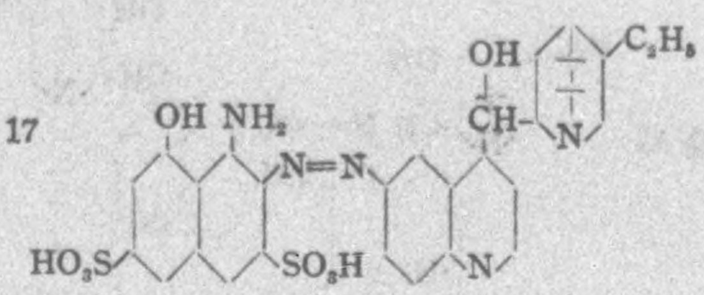

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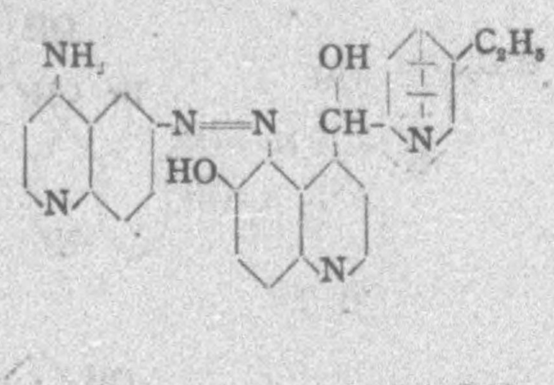

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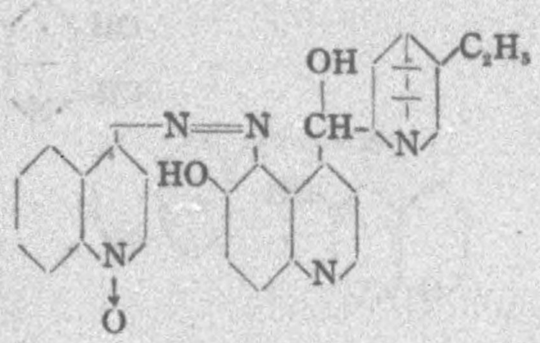

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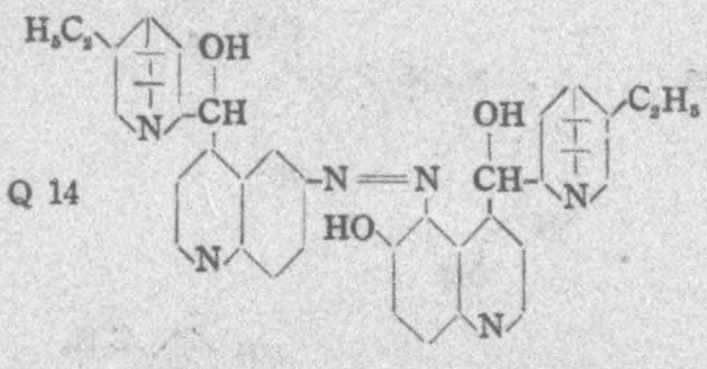

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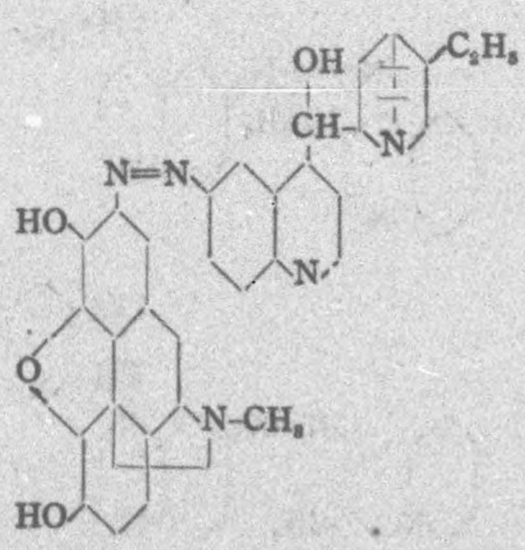

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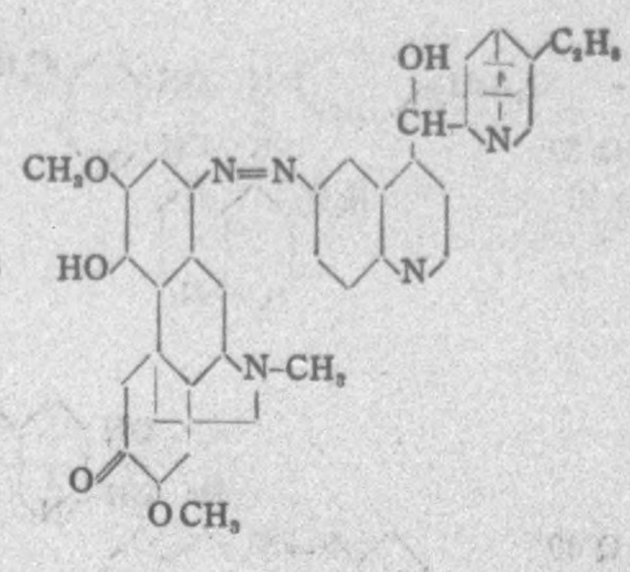

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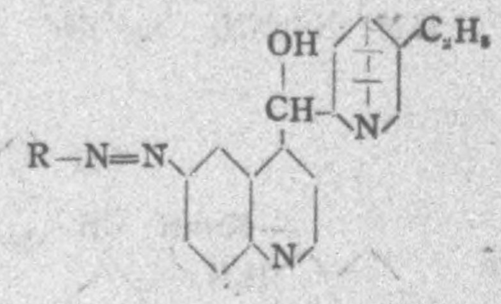

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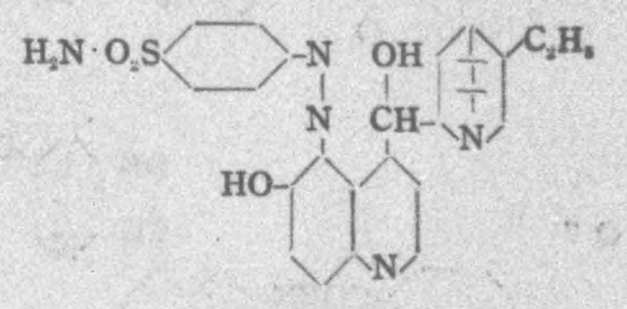

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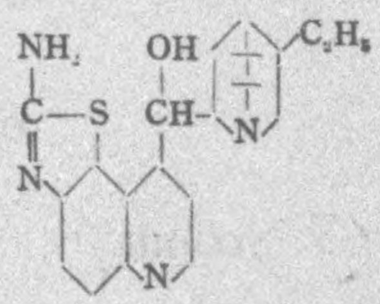

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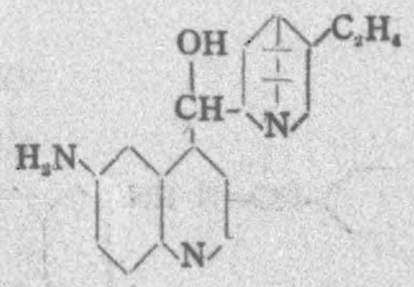

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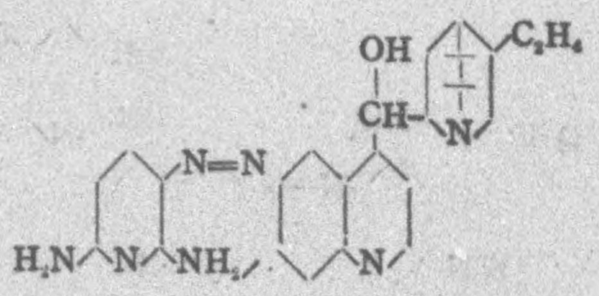

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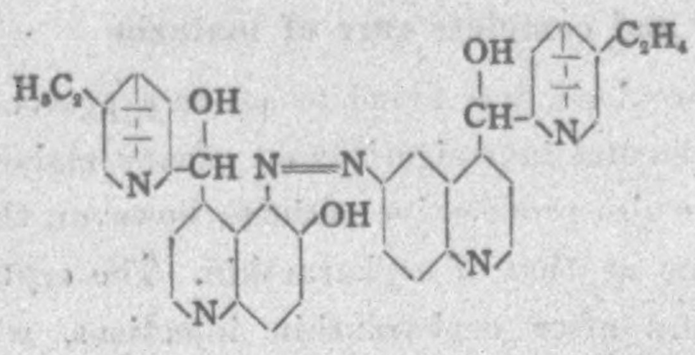
Q 35



Q 44

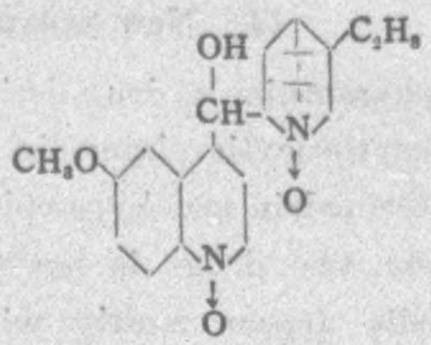


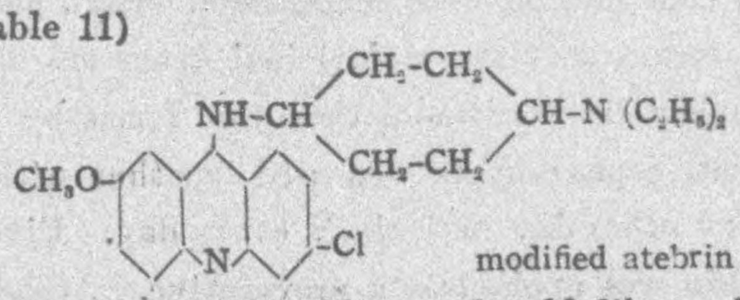
Table 10.

	M.E.D.	M.T.D.	M.T.D.: M.E.D.
Quinine hydrochloride	1:800	1:200	1:4
Atebrin	1:3000	1:100	1:30
Plasmoquine	1:50000	1:1500	1:30
Sulfa-dibromobenzene	1:200	1:50	1:4
Sulfadichlorobenzene	1:400	1:200	1:2
Q 4	1:800	1:50	1:16
Q 15	1:1600	1:100	1:16
Q 41	1:1600	1:200	1:8
Q 37	1:1600	1:400	1:4
Q 46	1:1600	1:100	1:16
Q 14	1:800	1:100	1:8
Q 11	1:800	1:100	1:8
Q 23	1:800	1:50	1:16
Atebrin (Asano)	1:3000	1:50	1:60
No. 19 (Taguchi)	1:800	1:100	1:8

M.E.D. . . . . Minimum effective dose M.T.D. . . . . Maximum tolerated dose

(I) Miscellaneous compounds

1) Modified atebrin was tested, in collabiration with Dr. M. Asano, which exhibited the same malaricidal power on canary malaria as atebrin. The drug is simpler in its manufacture than atebrin. The malaricidal action in five tertian cases was demonstrated with satisfactory results. (Table 11)



modified atebrin

2) Chemical compound of quininbisulfate and sulfadibromobenzene (produced by Dr. Taguchi) was examined in canary malaria with effective results. Consequently we applied this drug in five tertian cases, which showed satisfactory results. (Table 11)

Table 11

Drug	M.E.D.	M.T.D	M.T.D.: M.E.D.	
1) Asano	1: 3.000	1: 50	1:60	oral
	1:10 000	1:500	1:20	subcutan
2) Taguchi	1: 8000	1:100	1:8	oral

M.E.D. . . . . Minimum effective dose M.T.D. . . . . Maximum tolerated dose

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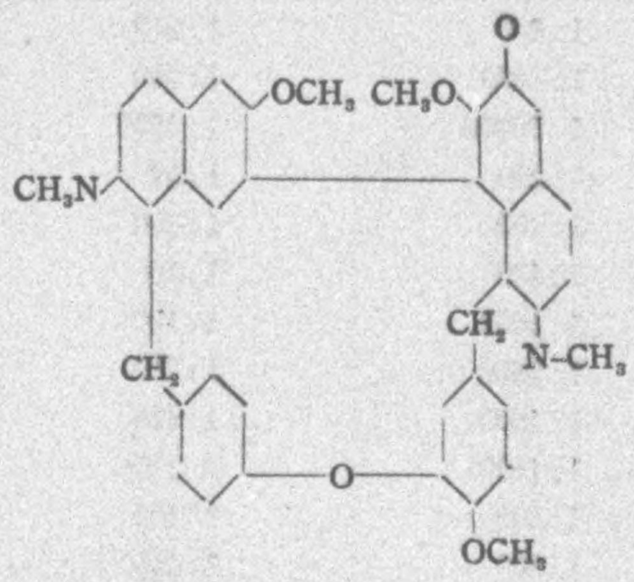
(J) New provocative drugs and complete cure of malaria

Cepharanthin, a drug designed for tuberculosis, was found to act as a provocative for extra-erythrocytic plasmodia in our experimental investigations on canary malaria.

Isotetrandrin and hypo-epistephanin have also provocative actions; however, the action of these two drugs is not quite the same as that of cepharanthin. The erythrocytic plasmodia appear mostly within 1–2 hours after cepharanthin injections, while the appearance of erythrocytic plasmodia is within about 20 hours after isotetrandrin and hypoepistephanin application.



Cepharanthin

The recently produced evidence that cepharanthin stimulates the endothelial cells explains the fact that the extra-erythrocytic forms are provoked out from the cells into the blood stream through endothelial stimulation.

Complete cure of malaria

It has been known that complete cure of malaria is difficult by means of quinine, atebrin, plasmoquine, etc., the reason being these drugs are not able to destroy the extra-erythrocytic forms of plasmodium. We, therefore, endeavored to bring about complete cure by cepharanthin in combination with malaricidal drugs, and our observations were fairly satisfactory. This means that the malaricidal drugs act on the erythrocytic forms after they have been changed from extra-erythrocytic forms by means of cepharanthin.

In the first experiment, cepharanthin was used together with quinine hydrochloride, in 5 to 7 successions every other day or 7 times every day. Five days after the therapy nos completed, cepharanthin was applied as a provocative. According to this experiment desirable results were not obtained, but the provocative action of cepharanthin was ascertained.

In the second experiment, we observed the behavior of erythrocytic plasmodium after either cepharanthin or hypoepistephanin was applied combined with quinine in a succession of 5 days. The plasmodium appeared wthin the erythrocytes from the 3rd to 5th day after the drugs were given. According to this observation, 5-day treatment followed by a 3-day interval seems to be better; therefore, for a complete cure this mode of treatment should be repeated several times. Accordingly 3 courses of treatment were applied in this experiment. As shown in table 12, cepharanthin, isotetrandrin and

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hypoepistephanin were used together with quinine hydrochloride. Ten days after these courses of treatment were carried out, cepharanthin was used as a provocative; 2 cases (66%) out of 3 showed complete cure of chronic malaria. (Table 12)

Table 12

	Cepharanthin + quinine			Isotetrandrin + quinine			Hypoepistephanin + quinine		
	1	2	3	1	2	3	1	2	3
1	+1	+1	–	–	+2	–	+1	+3	+6
	+1	+2	–	+1	–	–	+2	+1	+1
2	+1	–	–	–	–	–	–	–	+1
3	–	–	–	–	–	–	+1	–	–
4	–	–	–	–	–	–	–	–	–
5	–	–	–	–	–	–	–	+1	–
	Three days interval								
1	–	+1	–	+1	–	–	+1	–	–
2	–	–	–	–	–	–	+1	–	–
3	–	–	–	+1	–	–	–	+2	–
4	–	–	–	–	–	–	–	–	–
5	–	–	–	–	–	–	–	–	–
	Three days interval								
1	–	–	–	–	–	–	–	–	
2	–	–	–	–	–	–	–	–	
3	–	–	–	–	–	–	+1	–	
4	–	–	–	+4	–	–	+2	–	
5	–	–	–	+1	+1	+1	+1	+2	
	After ten days cepharanthin was given								
1	–	–	–	+1	+1	+2			
2	–	–	+1						
3	–	–							

(+2 ..... two parasites found in one hundred fields.
– ..... no parasites found.)

In the third experiment, atebrin and plasmoquine were used together with cepharanthin. Three cases (60%) out of 5, which were treated with cepharanthin and with atebrin or plasmoquine showed complete cure; however, 3 cases in which cepharanthin was not applied but atebrin alone was used, showed incomplete cure by provocative cepharanthin. (Table 13)

Table 13

	Atebrin			Atebrin + cepharanthin				
	1	2	3	1	2	3	4	5
1	–	+2	–	+4	–	+2	+1	+1
	–	+4	+8	–	–	–	+1	–
2	–	+4	+6	–	–	–	–	–
3	–	+2	–	–	–	–	–	–
4	–	+2	–	–	–	–	–	–
5	–	–	–	–	–	–	–	–
	Three days interval							
1	–	–	–	–	–	–	–	–
2	–	–	–	–	–	–	–	–
3	–	–	–	–	–	–	–	–
4	–	–	–	–	–	–	–	–
5	–	–	–	–	–	–	–	–

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				Three days interval				
1	−	−	−	−	−	−	−	−
2	−	−	−	−	−	−	−	−
3	−	−	−	−	−	−	−	−
				After seven days cepharanthin was given				
1	+1	+1	+1	−	−	−	−	−
2	+1	+1	+1	−	−	−	+1	−
3	+1	+1	+1	+1	−	−	+1	−

Two (60%) out of 3 cases, which were tried with cepharanthin and plasmoquine, showed complete cure; however, 3 cases treated only with plasmoquine showed incomplete cure. (Table 14)

Table 14

	Plasmoquine			Plasmoquine + cepharanthin		
	1	2	3	1	2	3
1	+1	+1	+1	+1	+1	+1
	−	−	−	−	−	+4
2	−	−	−	−	−	+9
3	−	−	−	−	−	−
4	−	−	−	−	−	−
			Five days interval			
1	+3	−	−	−	−	−
2	−	−	−	−	−	−
3	−	−	−	−	−	−
4	+2	−	−	−	−	−
5	−	−	−	−	−	−
			Five days interval			
1	−	−	−	−	−	−
2	−	−	−	−	−	−
3	−	−	−	−	−	−
4	−	−	−	−	−	−
5	−	−	−	−	−	−
			After seven days cepharanthin was given			
1	+1	−	+1	−	−	+1
2	−	−		−	−	
3		+1		−	−	

Conclusion: From the above experiments it is concluded that the complete cure of malaria is expected when cepharanthin together with malaricidal drugs are employed.

II. Chemotherapy of Spirochaetosis.

The efficacy of a number of drugs against spirochaetosis recurrentis (duttoni) in white mice was tested in our laboratory. We found that sulfapyridine, sulfathiazole and sulfamethylthiazole showed some activity on spirochaetes.

(1) Sulfonamide compounds.

Forty three sulfonamide compounds were examined in experimental spirochaetosis recurrentis of mice. (Table 15, 16)

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Table 15

Sulfanilamide
Sulfanilguanidine
p-Aminobenzolsulfonacetamide
Diseptal A
Diseptal B
Diseptal C
p-(p-Aminophenyl)-benzene-sulfonamide
Sulfanilanilide
1-Sulfanilamido-3, 5-dibromobenzene
1-Sulfanilamido-3, 5-dichlorobenzene
Sulfapyridine
2-(4′-Amino-3′, 5′-dibromo-benzene sulfonamido)-pyridine
2-Sulfanilamido-6-methyl-pyridine
3-Sulfanilamido-2, 6-lutidine
2-Sulfanilamido-5-chloro-pyridine
2-Sulfauilamido-5-bromo-pyridine
2-Sulfanilamido-5-amino-pyridine
Sulfathiazole
Sulfamethylthiazole
2-(4′-Amino-3′, 5′-dibromobenzene-sulfonamido)-5-bromo-thiazole
4-Sulfanilamido-1-phenyl-2, 3-dimethyl-5-pyrazolone
2-Sulfanilamido-benzothiazole
N-Dimethylsulfanilamide
Marfanilamide
3-Carboxy-4-aminoazobenzene-4′-sulfonamide
Rubiazol
Proseptasine
2-(N⁴-Sulfanilylsulfanilamido)-4-methylthiazole
N′-Dimethyl-N⁴-(N′⁴-sulfanilylsulfanil)-sulfanilamide
Phonocasil
2-Sulfanilamido-6-methoxy-benzothiazole
2-Sulfanilamido-6-ethoxy-benzothiazole
2-Sulfanilamido-5-bromo-benzothiazole
2-Sulfanilamido-6-choloro-benzothiazole
7-(4′-Sulfonamidophenylazo)-6-hydroxy-benzothiazole
7-(4′-Sulfonamidophenylazo)-2-methyl-6-hydroxy-benzothiazole
2-Sulfanilamido-pyridine
2-Sulfanilamido-4-methylpyrimidine
2-Sulfanilamido-4, 6-dimethyl-pyrimidine
Calco-acimidone
1-Sulfanilamido-4-bromo-naphthalene
1-Sulfanilamido-5, 8-dibromo-naphthalene

Table 16

Dates	Sulfapyridine 1 2 3 4 5 6	Sulfathiazole 1 2 3 4 5 6	Sulfamethylthiazole 1 2 3 4 5 6
Doses of drugs used	↓ ↓ ↓	↓ ↓ ↓	↓ ↓ ↓
0 03 g.	Sp infected − − − − −	Sp infected − − − − −	Sp infected − − − − −
0 02	− − − − −	− − − − −	− − − − −
0.01	+ − − − −	+ − − − −	+ ++ +++ +++ +++
0.005	+ ++ +++ +++ +++	+ ++ +++ +++ +++	+ ++ +++ +++ +++
Control	+ ++ +++ +++ +++	+ ++ +++ +++ +++	+ ++ +++ +++ +++

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The activity of sulfapyridine is the same as that of sulfathiazole but sulfamethylthiazole has less action than these two drugs.

(2) Diphenylsulfone and approximate compounds.

Eight compounds were tested with negative results. (Table 17)

Table 17

4, 4′-Diamidino-stilbene
4, 4′-Diamidino-diphenylsulfone
4, 4′-Diamidino-diphenylsulfoxyde
4, 4′-Diguanidino-diphenylsulfone
4 4′-Diguanidino-diphenylsulfoxyde
4, 4′-Diguanidino-diphenylsulfide
Promin
4-Aminophenyl-3′-aminopyridyl-(6′)-sulfone-N, N′-didextrose sulfonate

III. Chemotherapy of Trypanosomiasis

Experimental chemotherapy of trypanosomiasis (*Trypanosoma gambiense*) in white mice was carried out. It has been known that the effective drugs against trypanosomiasis of mice show trypanocidal action in human cases as well, and some effect in leishmaniasis. We examined three groups of chemical compounds in our investigation.

(a) Antimony compounds

New antimony compounds made by Dr. K. Tsuda were examined and compared with fouadin and neostibosan. (Table 18, 19) Nos. 104, 105, 106 and 110 seemed to be available for practical use.

Table 18

No. 101 8-Oxychinoline-5, 7-disulfonate sodium antimony compound
No. 102 8-Oxychinoline-5, 7-disulfonate diethylamine antimony compound
No. 103 8-Oxychinoline-5-sulfonate-sodium antimony compound
No. 104 7-Iodo-8-oxychinoline-5-sulfonate sodium antimony compond
No. 105 7-Bromo-8-oxychinoline-5-sulfonate sodium antimony compound
No. 106 7-Chloro-8-oxychinoline-5-sulfonate sodium antimony compound
No. 109 7-Amino-8-oxychinoline-5-sulfonate sodium antimony compound
No. 110 7, 8-Dioxychinoline-5-sulfonate sodium antimony compond

Table 19

Drugs	M.T.D.	M.E.D.	M.C.D.	$\frac{\text{M.T.D.}}{\text{M.E.D.}}$	$\frac{\text{M.T.D.}}{\text{M.C.D.}}$
No. 101	1.5 mg.	1.0 mg.	1.5 mg.	1:1.5	1:1
No. 102	1.0	1.0	—	—	—
No. 103	0.2	0.2	—	1:1	—
No. 104	0.8	0.13	0.13	1:6.1	1:6.1
No. 105	0.5	0.13	0.25	1:3.8	1:2
No. 106	0.6	0.15	0.25	1:4	1:2.4
No. 109	2.5	2.5	—	1:1	—
No. 110	0.9	0.35	0.4	1:2.6	1:2.2
Fouadin	6.4	0.4	0.8	1:1.6	1:8
Neostibosan	25.0	8.0	8.0	1:3.1	1:3.1

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(b) Sulfonamide compounds

Forty one sulfonamide compounds tested had no action on trypanosome infection. (Table 20)

Table 20

Sulfanilamide
Sulfanilylguanidine
p-Aminobenzolsulfonacetamide
Diseptal A
Diseptal B
Diseptal C
p-(p-Aminophenyl)-benzene-sulfonamide
Sulfanilanilide
1-(Sulfanilamido)-3, 5-dibromobenzene
1-(Sulfanilamido)-3, 5-dichlorobenzene
Sulfapyridine
2-Sulfanilamido-6-methylpyridine
3-Sulfanilamido-2 6-lutidine
2-Sulfanilamido-5-chloropyridine
2-Sulfani amido-5-bromopyridine
Sulfathiazole
Sulfanilamido-4-methyl-5-carbathoxy-thiazole
4-Sulfanilamido-1-phenyl-2, 3-dimethyl-5-pyrazolone
2-Sulfanilamido-benzothiazole
N-Dimethylsulfanilamide
Marfanilamide
Prontosil rubrum
3-Carboxy-4-aminoazobenzene-4′-sulfonamide
Rubiazol
Proseptasine
2-(N-Sulfanilylsulfanilamido)-4-methyl-thiazole
N′-Dimethyl-N-(N′-sulfanilylsulfanil)-sulfanilamide
2-(N⁴-(N′⁴-Sulfanilyl-sulfanilyl)-sulfanilamido)-4-methylthiazole
N′-Dimethyl-N⁴-(N′⁴-(N″⁴-sulfanilyl-sulfanilyl)-sulfanilyl)-sulfanilamide
Phonocasil
2-Sulfanilamido-6-methoxy-benzothiazole
2-Sulfanilamido-6-ethoxy-benzothiazole
2-Sulfanilamido-5-bromo-benzothiazole
2-Sulfanilamido-6-chloro-benzothiazole
7-(4′-Sulfonamidophenylazo)-6-hydroxy-benzothiazole
7-(4′-Sulfonamidophenylazo)-2-methyl-6-hydroxy-benzothiazole
2-Sulfanilamido-pyrimidine
2-Sulfanilamido-4-methylpyrimidine
2-Sulfanilamido-4, 6-dimethylpyrimidine
Calco-acimidon

(c) Diphenylsulfone and approximate compounds

Several new compounds, submitted by Dr. S. Sugasawa, were examined. The effect of diamidino-stilbene against *Trypanosoma rhodesiense* had already been observed by Yorke (1940). We tested the activity of diamidino-stilbene on *Trypanosoma gambiense* with the same results as those which he had observed.

There are still newer compounds which were found to have trypanocidal action according to our experiments. (Table 21, 22)

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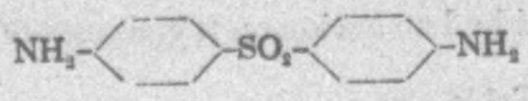
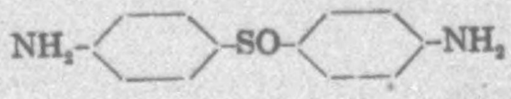
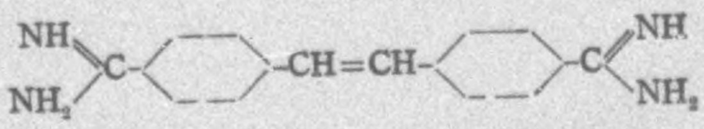
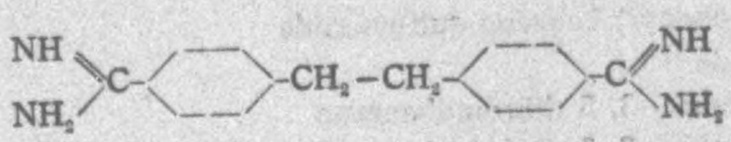
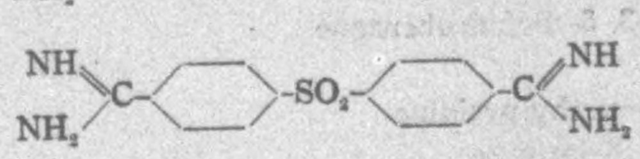
Table 21

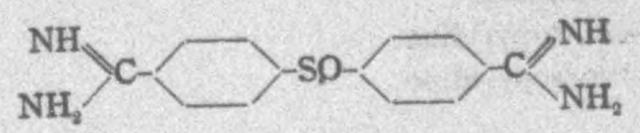
D 1 .  non-effective

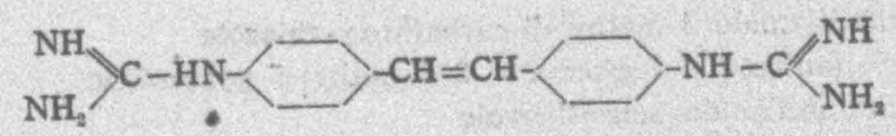
D 2  "

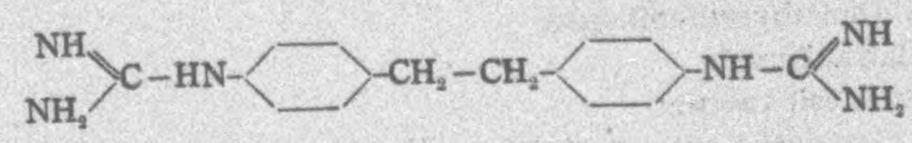
D 3  effective

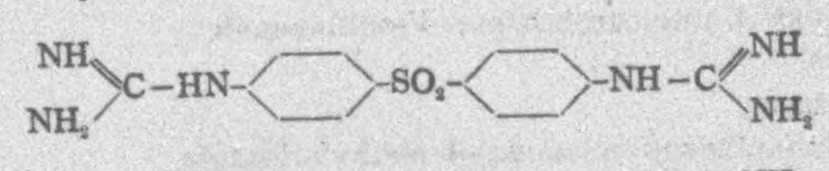
D 4  little effective

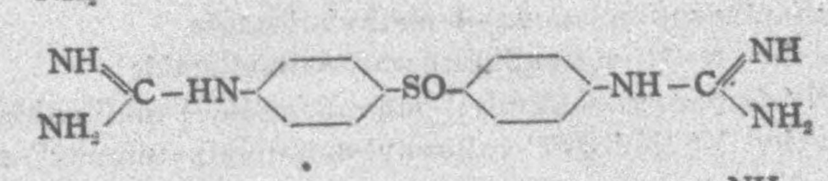
D 5  non-effective

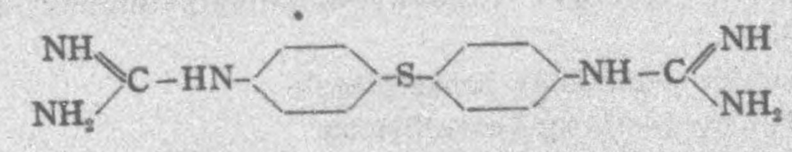
D 6  "

D 7  effective

D 8  "

D 9  "

D10  "

D11  "

(These drugs are shown in table 8)

Table 22

Drugs	M.L.D.	M.T.D.	M.E.D.	M.C.D.	$\frac{\text{M.T.D.}}{\text{M.C.D.}}$
D 1	—	—	—	—	—
D 2	—	—	—	—	—
D 3	1.5 mg	1 3 mg	0 004 mg	0.05 mg	1 : 26
D 4	0 9	0.8	0 7	—	—
D 5	2 5	2.0	—	—	—
D 6	1.5	1.0	—	—	—
D 7	1 2	1.0	0.5	?	—
D 8	0 6	0.5	0.2	?	—
D 9	0 9	0.8	0 3	0 6	1 : 1.3
D 10	1 8	1.5	0 2	0.3	1 : 5
D 11	0 4	0 3	0 05	0.3	1 : 1

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IV. Chemotherapy of Leishmaniasis

(1) Diphenylsulfone and approximate compounds.

From this work we concluded that 4,4′-diguanidino-diphenylsulfoxyde is the most readily availble drug for trypanosomiasis.

Accordingly, we applied this new drug in human leishmaniasis, since the drug which has trypanocidal activity shows also some action against kala-azar. In one course a total dose 1.28 g. of 4,4′-diguanidino-diphenylsulfoxyde was given intravenously into nine human kala-azar cases for 3 weeks on every other day; eight out of these cases demonstrated fairly good results. Thus a curative result will be obtained by repeating the administration of this drug, but leucopenia seems to be an attendant conflication. Therefore we are endeavoring to change the nature of this drug.

(2) Sulfonamide compounds.

Ten sulfonamide compounds were tested in kala-azar infection of striped squirrel, but no effect was found.

(3) Antimony compounds.

Nos. 104, 110 were tested in squirrel kala-azar with no effect but neostibosan was tried as a control with complete cure of the infection. This means the pentavalent antimony is more curative than the trivalent compounds.

V. Chemotherapy of Entamoebiasis

For the purpose of examining drugs we used cultivated *Entamoeba histolytica* in vitro. If the drug prevents the growth of amoebae, we suppose it to be an effective drug against amoebiasis. Through our investigations the well-known drugs, emetine hydrochloride and yatren, were used for control.

(1) Quinoline derivatives.

Yatren and other compounds were tested, with a result that Nos. 104, 105, 106 were three times as effective as yatren. (Table 23, 24)

Entamoeba histolytica was cultivated in Dobell-Laidlaw's medium, the fluid containing drugs in several concentrations. The drugs used are as follows:

Yatren 7-Jodo-8-oxyquinoline-5-sulfonate
No. 104 7-Jodo-8-oxyquinoline-5-sulfonate sodium antimony compound
No. 105 7-Bromo-8-oxyquinoline-5-sulfonate sodium antimony compound
No. 106 7-Chloro-8-oxyquinoline-5-sulfonate sodium antimony compound
No. 110 7, 8-Dioxyquinoline-5-sulfonate sodium antimony compound

Table 23 Growth of *E. histolytica*

Drug	Concentration	24 hrs	48 hrs	72 hrs	resu ts
Yatren	1: 1.000	−	−	−	effective
	1: 2.000	+	−	−	〃
	1: 3.000	++	+	+	non-effective
	1:10.000	++	++	+	〃

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Drug	Concentration	24 hrs	48 hrs	72 hrs	results
No. 104	1: 3.000	−	−	−	effective
	1: 5 000	−	−	−	〃
	1: 7 000	+	±	−	〃
	1:10.000	+	+	+	non-effective
No. 105	1: 3 000	−	−	−	effective
	1: 5 000	−	−	−	〃
	1: 7 000	+	−	−	〃
	1:10.000	+	+	+	non-effective
No. 106	1: 3 000	−	−	−	effective
	1: 5 000	−	−	−	〃
	1: 7 000	+	±	−	〃
	1:10 000	+	+	+	non-effective
No. 110	1: 1 000	+	+	+	〃
	1: 3.000	++	++	+	〃
Control		++	+++	+++	

Table 24

	1:1000	1:2000	1:3000	1:5000	1:7000	1:10000	1:50000	1:100000
Emetine						○	△	×
Osvarsan	○	○	△					
Yatren	○	○	△					
No. 104			○	○	○	△		
No. 105			○	○	○	△		
No. 106			○	○	○	△		
No. 110	△		×					

○.....effective △.....slightly effective ×.....non-effective

(2) Sulfonamide compounds

Thirty one sulfonamide compounds were tested; 2 of these seemed to be practically effective for amoebiasis. (marked O in Table 25, 26)

Table 25

Sulfanilamide
Sulfanilylguanidine
Diseptal A
Diseptal B
Diseptal C
p-(p-Aminophenyl)-benzene-sulfonamide
Sulfanilanilide
○ 1-Sulfanilamido-3, 5-dichlorobenzene
Sulfapyridine
2-Sulfanilamido-5-chloropyridine
Sulfathiazole
2, 3-Dimethyl-5-pyrazolone
1-Sulfanilamido-3, 5-dibromobenzene
3-Sulfanilamido-quinoline

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2-Sulfanilamido-benzothiazole
N-Dimethylsulfanilamide
Marfanilamide
○ 3-Carboxy-4-aminoazobenzene-4′-sulfonamide
Rubiazol
Proceptasine
N′-Dimethyl-N⁴-(N′⁴-sulfanylsulfanil)-sulfanilamide
N′-Dimethyl-N⁴-(N′⁴-(N′′⁴-sulfanylsulfanilyl)-sulfanilyl)-sulfanilamide
Phonocasil
2-Sulfanilamido-pyrimidine
3, 5-Dibromo-sulfanilamide
2-Sulfanilamido-5-amino-pyridine
Calcoacimidon
p-Aminobenzol-sulfonacetamide
2-Sulfonamido-5-bromo-benzothiazole
1-Sulfanilamido-4-bromo-naphthalene
1-Sulfanilamido-5,8-dibromo-naphthalene

Table 26

Drug \ Concentration	1 : 200	1 : 300	1 : 500	1 : 1,000	1 : 2,000	1 : 3,000	1 : 5,000	1 : 10,000	1 : 50,000	1 : 100,000
Sulfanilamide	○	△	×							
1-Sulfanilamido-3, 5-dibromobenzene	○	△	×							
1-Sulfanilamido-3, 5-dichlorobenzene	○	○	○	○	×	×	×	×		
3-Carboxy-4-amino azobenzene-4′-sulfonamide	○	○	○	△	×	×	×	×		
Phonocasil	○	○	△							
2-Sulfonamido-5-bromo-benzothiazole	○	△	×							
Proceptasine	○	△	×							
Yatren				○	○	△				
Emetine hydrochloride								○	△	×

○ Effective △ Slightly effective × Non-effective

(3) Miscellaneous compounds

Nine miscellaneous compounds were tested resulting non-effective.

Phenothiazine
4, 4′-Diamidino-stilbene
4, 4′-Diamidino-diphenylsulfone
4, 4′-Diamidino-diphenylsulfoxyde
4, 4′-Diguanidino-diphenylsulfone
4, 4′-Diguanidino-diphenylsulfoxyde
4, 4′-Diguanidino-diphenylsulfide
Promin
4-Aminophenyl-3′-aminopyridyl-(6′)-sulfone-N, N′-didextrose sulfonate

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VI. Chemotherapy of Trichomoniasis

Trichomonas hominis, cultivated in Dobell-Laidlaw's medium, was used for testing effectiveness of the drugs. The drugs were diluted into the liquid part of the media, in which *T. hominis* was cultivated.

(1) Quinoline derivatives

Five quinoline derivatives were used, emetine hydrochlide and osvarsan were used for comparison. Nos. 104, 105, 106 were ten times as effective as yatren and little more than emetine hydrochloride. Therefore these three seem to be practically useful against *Trichomoniasis. hominis.* (Table 27)

Yatren 7-Jodo-8-oxyquinoline-5-sulfonate
No. 104 7-Jodo-8-oxyquinoline-5-sulfonate sodium antimony compound.
No. 105 7-Bromo-8-oxyquinoline-5-sulfonate sodium antimony compound
No. 106 7-Chloro-8-oxyquinoline-5-sulfonate sodium antimony componnd
No. 110 7,8-Dioxyquinoline-5-sulfonate sodium antimony compound

Table 27

	1:1,000	1:2,000	1:3,000	1:5,000	1:7,000	1:10,000	1:20,000
Emetine						△	×
Osvarsan	○	×					
Yatren	○	×					
No. 104	○	○	○	○	○	○	×
No. 105	○	○	○	○	○	○	×
No. 106	○	○	○	○	○	○	×
No. 110	○	○	○	△	×		

Table 28 Sulfonamide

Sulfanilamide
Sulfanilylguanidine
Diseptal A
Diseptal B
Diseptal C
p-(p-Aminophenyl)-benzene-sulfonamide
Sulfadibromobenzene
Sulfadichlorobenzene
Sulfapyridine
Sulfachloropyridine
Sulfathiazole
Sulfamethylthiazole
4-Sulfanilamido-1-phenyl-2, 3-dimethyl-5-pyrazolone
2-Sulfanilamido-quinoline
2-Sulfanilamido-benzothiazole
N'-Dimethylsulfanilamide
Marfanilamide
Prontosil rubrum
2,4-Oxyazobenzene-4'-Sulfonamide
3-Carboxy-4-aminoazobenzene-4'-sulfonamide
Rubiazole
Proseptasine

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N′-Dimethyl-N⁴-(N′⁴-sulfanilylsulfanil)-sulfanilamide
2-(N⁴-(N′⁴-sulfanilyl-sulfanilyl)-sulfanilamido)-4-methylthiazole
N′-Dimethyl-N⁴-(N′⁴-(N″⁴-sulfanilyl-sulfanilyl)-sulfanilyl)-sulfanilamide
Phonocasil
2-Sulfanilamido-5-bromo-benzothiazole
2-Sulfanilamido-pyrimidine
2-Sulfanilamido-4-methyl-pyrimidine
2-Sulfanilamido-4, 6-dimethyl-pyrimidine
2-Sulfanilamido-5-amino-pyridine
Calco-acimidon
p-Aminobenzol-sulfonacetamide
1-Sulfanilamido-5-bromo-naphthalene
1-Sulfanilamido-4-bromo-naphthalene
1-Sulfanilamido-5, 8-dibromo-naphthalene

(2) Sulfonamide compounds

Thirty six sulfonamide compounds were tested, but none of them seemed to be of valve for trichomoniasis. (Table 28)

(3) Diphenylsulfone and approximate compounds

Eleven drugs were tested against *Trichomonas hominis* in culture. D3 and D7 were found to be more potent than others, these two stilbene compounds, appearing to be effective against *Trichomonas*. Stilbene guanidine is more effective than stilbene amidine. (Table 29)

Summary

The researches on the chemotherapy of protozoal infections, malaria, spirochetosis, trypanosomiasis, leishmaniasis, entamebiasis and trichomoniasis, were carried out in this laboratory.

1. Out of 53 sulfonamide compounds, sulfadibromobenzene and sulfadichlorobenzene were found effective on canary malaria; sulfadibromobenzene was applied on human tertian malaria with desirable results. Eight quinine derivatives, out of 42 tested, exhibited a malaricidal action on canary malaria, and 6-amino-dihydro-cinconidine showed satisfactory results in human tertian cases.

   Dr. Asano's atebrin was found to be as effective as other types of atebrin on canary malaria and human tertian cases.

   Chemical compoune of quininebisulfate and sulfadibromobenzene were also examined with effective result in both canary and human malaria.

   Cepharanthin was found to be a provocative drug which can be applied for the purpose of complete cure of malaria when accompanied by malaricidal drugs.
2. Out of 43 sulfonamide compounds examined, sulfapyridine, sulfathiazole and sulfamethylthiazole were found to be effective in mice spirochetosis recurrentis.

   7-Jodo-8-oxyquinoline-5-sulfonate sodium antimony compound
   7-Bromo-8-oxyquinoline-5-sulfonate sodium antimony compound
   7-Chloro-8-oxyquinoline-5-sulfonate sodium antimony compound
   7, 8-Dioxyquinoline-5-sulfonate sodium antimony compound

   showed satisfactory results in mice trypanosomiasis.

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Table 29

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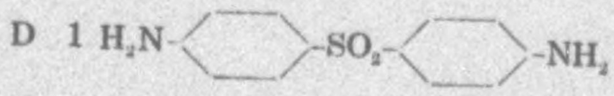
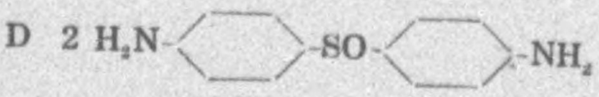
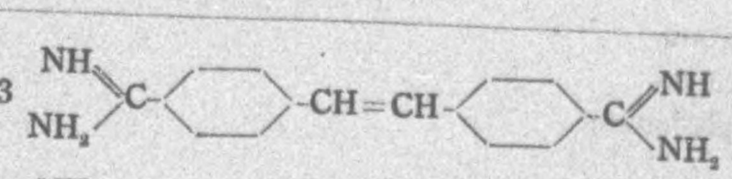
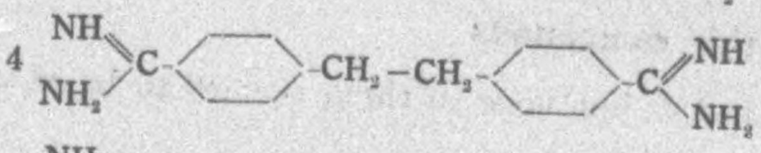
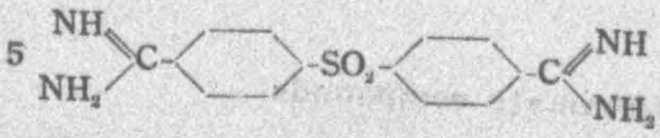
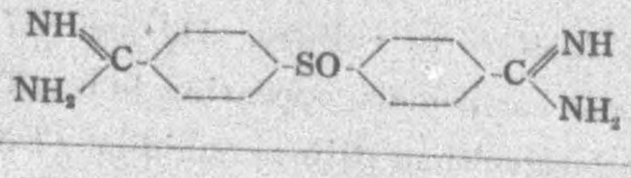
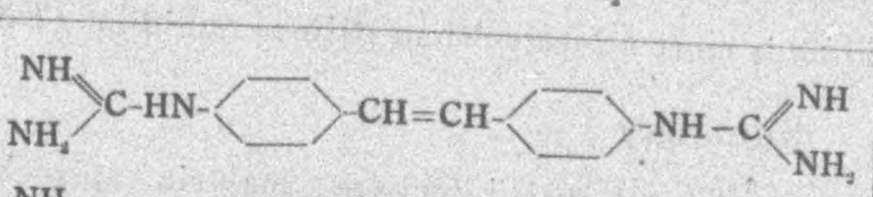
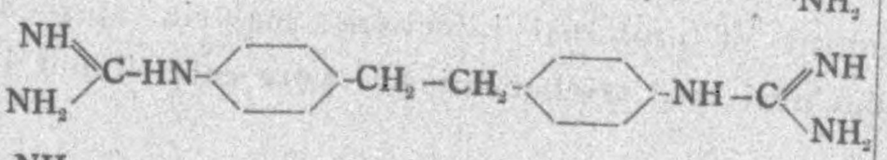
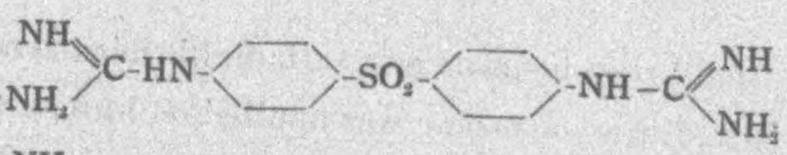
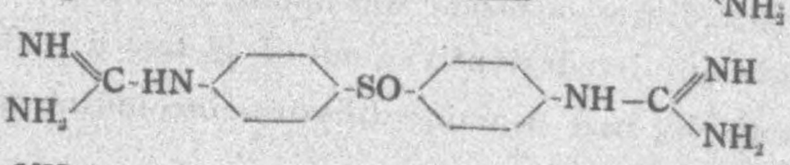
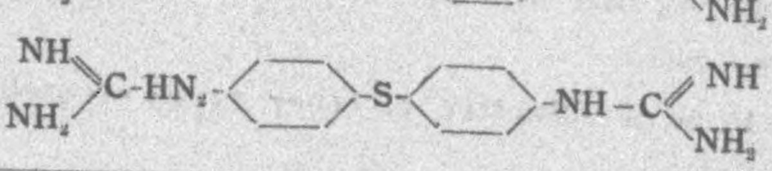
D 1 	×					
D 2 	×					
D 3 	○	○	○	○	△	×
D 4 	○	○	△			
D 5 	△	×				
D 6 	△	×				
D 7 	○	○	○	○	○	△
D 8 	○	○	×			
D 9 	×					
D 10 	○	×				
D 11 	○	△				

4. Diguanidino-diphenylsulfoxyde was applied in human kala-azar cases with good effects.
5. 7-Jodo-8-oxyquinoline-5-sulfonate sodium antimony compound, 7-bromo-8-oxyquinoline-5-sulfonate sodium antimony compound and 7-chloro-8-oxyquinoline-5-sullonate sodium antimony compound were found effective for entamebiasis.
6. Three quinoline derivatives, 7-jodo-8-oxyquinoline-5-fulfonate sodium antimony compound, 7-bromo-8-oxyquinoline-5-sulfonate sodium antimony compound and 7-chloro-8-oxyquinoline-7-sulfonate sodium antimony compound were found effective in trichomoniasis. Diamidino-stilbene and diguanidino-stilbene also showed satisfactry results in trichomoniasis.

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STUDIES ON THE MECHANISM OF THE AGGLUTINATION
I. ON THE LATTICE THEORY

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The field of the immunology is so extensive and the mechanism of the immunological reactions is so complex that no one has found it possible to induce an inclusive theory.

It seems to be too adventurous to explain the mechanism of the infection and prevention now with the aid of the chemistry and physics. But in the field of the simplest reactions, precipitation and agglutination, the recent informations of the chemistry seem to be available and the mechanism of these simple reactions will be useful for the study of the more complex one, as the most important phenomenon, the specific combination, is common to all reactions.

At about the time when I have undertaken this work (1939), a splendia theory was advanced by Pouling (1) (1940).

Almost all our experiments were performed just before the war, but could not be published owing to the difficult communication in the recent several years. Our results seem to almost conform to Pauling's theory, and contribute to the complex immunological reactions.

In this report an experiment to test Marrack's Hypothesis (2) (Lattice theory) is described. Marrack's Lattice theory has been confirmed by several workers, Heidelberger (3), Topley, Wilson, and Duncan (4), but challenged by Abramson (5), Hooker and Boyd (6), and Haurowitz (7).

Though the experiment by Topley and others is very simple the result has a great meaning in deciding whether there are two stages, specific combination and non-specific aggregation, or only one specific stage in the course of agglutination.

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Topley and others found that in the mixed agglutination of two kinds of bacteria, showing no antigenic relationship, relatively large aggregates were formed, each consisting entirely of one kind of bacteria.

I not only confirmed this experiment, but also found that if two kinds of bacteria had the common antigen, the aggregates were formed by both of them.

Experimental

I) The structure of the aggregates of two kinds of bacteria, showing no antigenic relationship.

The combinations—Staphylococcus albus and Sallmonella cholerae suis, Staphylococcus albus, and B. dysenteriae (Komagome A)—were used. And the procedure was completely the same as of Topley and others.

As may be seen in Table 1 and Fig. 1—A and B, an aggregate was formed by only one kind of bacteria.

II) The structure of the aggregates of the two kinds of bacteria containing the common antigen.

To attach the common antigenic character to two kinds of bacteria, they were diazotized. After the bacteria were washed with physiological saline three times, 200 mg was suspended in 5 cc physiologcal saline added with 7.5 cc of 1N-Na_2CO_3 and then 1 cc atoxylazo-solution added drop by drop. During this process the mixture was cooled to 0°C. After 30 minutes, they were centrifuged and washed with physiological saline five times and the atoxyl-azo-bacteria were obtained.

Atoxyl-azo-solution was prepared as following: 1.5 g atoxyl was dissolved in 18 cc of 1N-HCl and 4.32 cc of 10% $NaNO_2$ was added and then the solution was diluted to 30 cc with distilled water.

Antiatoxyl-azo-antibody was prepared from the rabbit according to the description by Haurowitz, Kraus, and Marx (8).

Two kinds of atoxyl-azo-bacteria were mixed in physiological saline and the anti-atoxyl-azo-serum diluted with saline was added, then after agglutination took place they formed the mixed aggregates, as shown in Table 1 and Fig. 1—C and D. The formation of the mixed aggregates is not due to the non-specific combination, as the other experiment shows that if the bacteria and the other atoxyl-azo-bacteria are mixed and their corresponding antibodies are added, then they gather in the separate aggregates (Table 1).

Before these experiments it was confirmed that the agglutination of the atoxyl-azo-bacteria and atoxyl-azo-antibody is the immunologically specific one, as it was inhibited by the addition of the atoxyl-azo-tyrosine prepared by Pauli's method (9).

Discussion

The results shows that two kinds of bacteria A and B showing no antigenic relationship agglutinate in the separate aggregates, but on the contrary if they have an antigenic relationship they agglutinate in the mixed aggregates. If there were a stage of non-

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specific combination, then in all cases an aggregate would consist of two kinds of bacteria.

The antigen and antibody must bind themselves together specifically, and their compounds grow larger, binding specifically the other same compounds or the antigens and antibodies and at last the visible flocculi must be formed.

As I finished this experiment I was informed of the report by Wiesner and Herman (10) who reached the same conclusion by using other materials.

Table 1

The structure of the aggregates.

Combination of bacteria	Combination of antiserum	Aggregates
S. albus S. cholerae suis	Anti-Staphylococcus Anti-S. cholerae suis	separate
S. albus B. dysenteriae	Anti-Staphylococcus Anti-B. dysenteriae	separate
Atoxylazo-Staphylococcus Atoxylazo-S. cholerae suis	Anti-atoxyl	mixed
Atoxylazo-Staphylococcus Atoxylazo-B. dysenteriae	Anti-atoxyl	mixed
Atoxylazo-Enterococcus Atoxylazo-B. dysenteriae	Anti-atoxyl	mixed
Staphylococcus Atoxylazo-S. cholerae suis	Anti-Staphylococcus Anti-atoxyl	separate
Staphylococcus Atoxylazo-B. dysenteriae	Anti-Staphylococcus Anti-atoxyl	separate
S. cholerae suis Atoxylazo-Staphylococcus	Anti-S. cholerae suis Anti-atoxyl	separate
S. cholerae suis Atoxylazo-Enterococcus	Anti-S. cholerae suis Anti-atoxyl	separate
B. dysenteriae Atoxylazo-Staphylococcus	Anti-B. dysenteriae Anti-atoxyl	separate
B. dysenteriae Atoxylazo-Enterococcus	Anti-B. dysenteriae Anti-atoxyl	separate

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Fig. 1

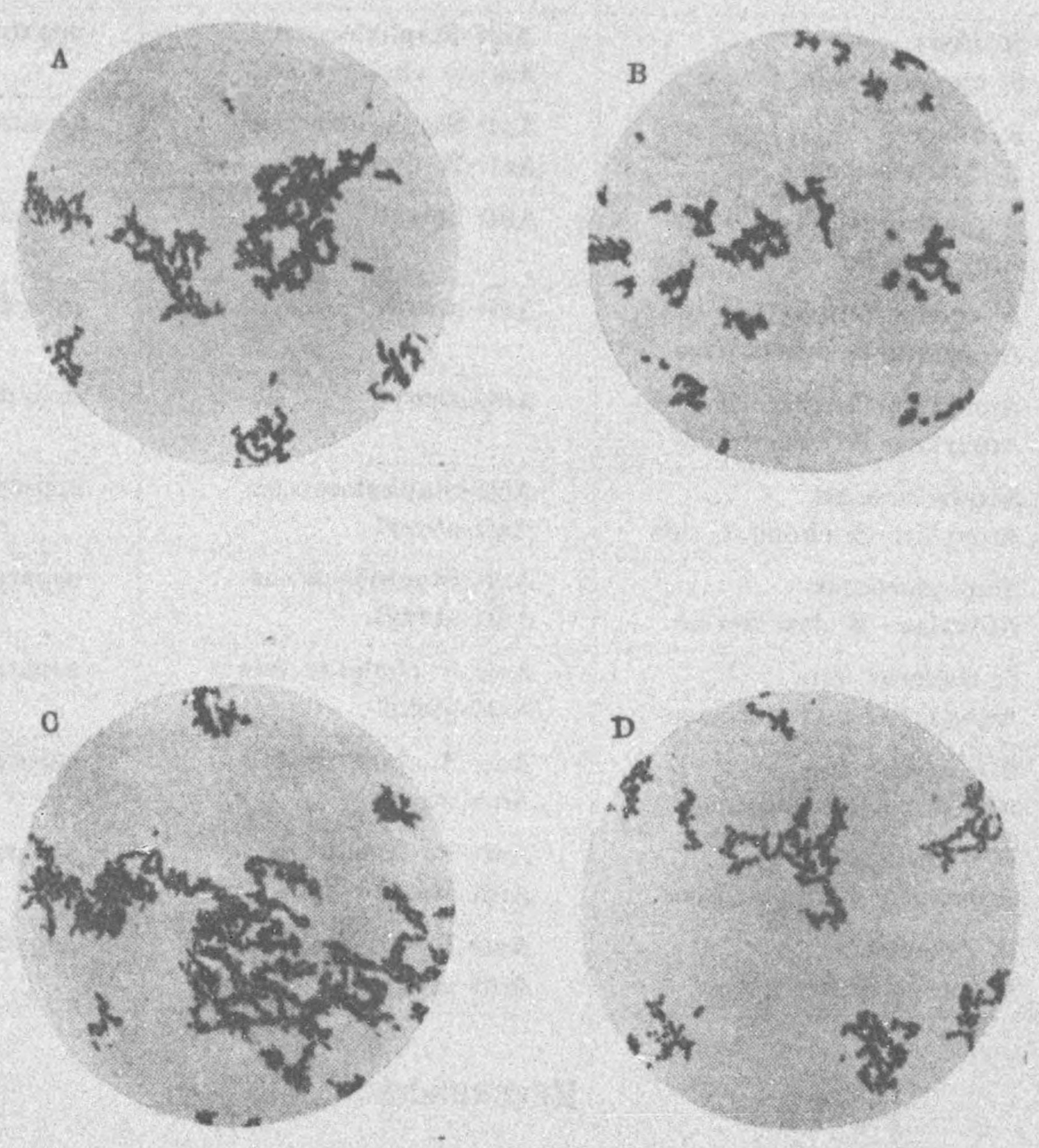
Structure of the aggregates.

A. S. cholerae suis + Anti-S. cholerae suis serum
Staphylococcus + Anti-Staphylococcus serum

B. B. dysenteriae + Anti-B. dysenteriae serum
Staphylococcus + Anti-Staphylococcus serum

C. Atoxylazo-Staphylococcus
Atoxylazo-S. cholerae suis } + Anti-atoxyl serum

D. Atoxylazo-B. dysenteriae
Atoxylazo-Staphylococcus } + Anti-atoxyl serum



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STUDIES ON THE MECHANISM OF THE AGGLUTINATION

II. On the Quantitative Relation between the Surface Area of the Bacteria and the Binding Agglutinin

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The previous simple experiment (1) suggests that in the course of the agglutination the bacteria and the agglutinin bind themselves specifically and then the antigen-antibody compounds grow larger, binding the others or the floating bacteria and agglutinin specifically. Now to find the structure of the bacteria-agglutinin compound in detail, the quantitative relation between the bacteria and the binding agglutinin was studied.

Many workers (2) reported that the quantity of the antibody could be estimated by measurung the nitrogen content of the antigen-antibody compound, and particularly Heidelberger (3—5) has published the splendid quantitative results. The quantitative relation between the surface area and the agglutinin experimentally obtained by us, not only confirms my previous experiment, but also suggests the configuration and the size of the antibody and further the place where the agglutinin attachs itself to the bacteria.

METHODS AND MATERIAL

I. Staphylococcus albus was used. Its 18 hour growth on the nutrient agar was suspended in saline, added with formaline 0.5%. After preserved 24 hours in ice box, centrifuged and washed with saline three times, and centrifuged once more prior to the experiment, suspended in saline and then the nitrogen content in every 1 cc was measured.

II. The antiserum against S. albus was prepared from the rabbit. The serum was heated 30 minutes at 56°C and preserved in the refrigerator. Before the experiment it was centrifuged 20 minutes at 6500 rounds per minute, in order to remove the precipitates which interfere with the estimation of the antibody.

III. The measuring method of the antibody nitrogen (Table 1). The volume of the bacterial suspension was correctly measured and then it was added with the antiserum diluted with physiological saline. This mixture was preserved 2 hours at 37°C and then 24 hours at 0°C. It was centrifuged and the precipitate was washed with physiological saline two times and then its nitrogen content (a) was measured by micro-kijeldahl. Simultaneously, instead of the anti-serum, the normal rabbit serum or physiological saline (any difference in the nitrogen content of the precipitates could not be observed between them) was added to the same quantity of the bacterial suspension, and the nitrogen content of the precipitate (b) was measured in the same way as described above. Then the antibody nitrogen (a—b) was calculated.

IV. The total antibody nitrogen of the antiserum. The nitrogen content of the antiserum and that after the adsorption of the antibody by the bacteria was measured and from their difference the antibody nitrogen of the antiserum was calculated. But as in this case the small quantity of the nitrogeneous substance was dissolved from the

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bacteria, so simultaneously the nitrogen dissolved from the bacteria was measured and the antibody nitrogen was checked by this value.

V. The radius of Staphylococcus. It was measured by three methods.

A) The weight of the bacteria dried at the room temperature was divided by the bacterial number. And from this weight of the one bacterium and the specific gravity of the dry bacteria, the radius could be calculated, as they are spherical.

B) From the sedimentation velocity of the bacteria, their radius was calculated. In this case their specific gravity was measured by observing the concentration of the saccharose solution in which the bacteria could not be precipitated by the centrifuge.

C) The radius was measured by electron-microscope. According to the method A, the radius was 0.32μ and the specific gravity of the dry bacteria was 1.40. The method C showed 0.3μ as the radius. Both the methods A and B measured the radius of the dry-bacteria, so they must conform to each other. This coincidence suggests that Staphylococcus is undoubtedly spherical. But the radius was measured as 0.35μ by the method B, and the specific gravity was 1.25. This difference between the values obtained by the method B, and the method A or C must be due to the fact that the bacteria loose the water while being dried and afterwards become smaller. If the dry bacteria which radius is 0.32μ and specific gravity 1.40 take the water until their specific gravity becomes 1.25, then their radius must become 0.37μ. This value (0.37μ) agrees fairly well with 0.35μ, experimentally obtained. As among the three methods the fluctuation was minimum in the method A, and besides in our experiment only the radius of the bacteria in suspended in saline was necessary, so in all our calculations 0.37μ was used as the radius. But the subsequent conclusion was completely the same, even though the radius 0.35μ was used.

Results and Discussion

I) The specifically precipitable nitrogen in the antiserum. The suspension of the Staphylococci was added with its antiserum, anti-cholera serum, anti-typhoid serum, or normal rabbit serum and nitrogen content of the precipitates was measured.

As in Table 1, the nitrogen content of the precipitates from the corresponding antiserum is undoubtedly different from the others and suggests that the difference of the nitrogen content between the precipitates from the corresponding antiserum and the others must be due to the agglutinin.

II) The nitrogen content of the agglutinin. The bacteria was suspended in the diluted antiserum and saline, centrifuged and washed with saline two times, and with ether, and dried. From the difference of their weights the quantity of the antibody was obtained. At the same time the nitrogen of the agglutinin adsorbed by the bacteria was measured. From the weight of the antibody and its nitrogen, the nitrogen content of the antibody was calculated. As shown in Table 2, it is about 13—14%. It suggests that the antibody is a kind of protein. But this estimation contains many errors, so in the subsequent calculations the following relation,

$$\text{Agglutinin weight} = \text{Agglutinin} - N \times 6.32$$

was used, according to Heidelberger (6).

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III) The relation between the surface area of the bacteria and the binding agglutinin.

The antiserum was diluted from 1:2.5 to 1:5120, and to 4 c.c of these various dilutions 1 c.c of the bacterial suspension was added and the agglutinin-nitrogen was measured as described above. From the results shown in Table 3, the following conclusion can be deduced.

1) In the range of the serum dilution where the agglutination is positive, the quantities of the binding agglutinin are not constant. The more agglutinin attaches to the bacteria, as the antibody concentration increases. If in the course of the agglutination, the whole surface of the bacteria were coated by a layer of the antibody and then these antibody-antigen compounds aggregated non-specifically, then the binding agglutinin content would be constant. On the contrary, the binding agglutinin content depends upon the agglutinin concentration, and the highest quantity of the binding agglutinin is about 15—27 times larger than the minimum quantity. It suggests that when only 1/15—1/27 of the bacterial surface is occupied by the agglutinin, the agglutination can take place. The results seem to conform to Marrack's Lattice theory (2). The same results can also be observed, in the report by Heidelberger (4, 5, 7).

2) The size of the agglutinin and the structure of the antigen-antibody compound.

The quantities of the binding antibodies are almost the same at the serum dilutions, 1:2.5 and 1:5, so it seems that in these cases the almost whole surface is occupied by the antibodies. If it is so, the thickness of the antibody layer (fig. 1) can be calculated as follows:

$$4/3\pi(r+h)^3-4/3\pi r^3=\frac{N\times 6.32\times 0.745}{10^3\times \text{bacterial number}}$$

(r is the radius of the bacteria in cm, h the thickness of the antibody layer in cm, N the nitrogen of the binding antibody in mg, 0.745 the specific gravity of the antibody according to Heidelberger.)

From the results shown in Table 3, h is about 19 mμ. But the antibody must have a certain structure, and among the antibodies attaching to the bacteria there must be many crevices, so the thickness of the antibody layer must be larger than the calculated value (19 mμ).

The molecular weight of the antibodies in the rabbit serum against Pneumococcus polysaccharid (8), egg albumin (8), and dye-protein (9) were determined as 156000. It is fairly probable that the antibody againts Staphylococcus has also the same molecular weight. Then if the antibody were spherical, its diameter would be 7.2 mμ. It is much smaller than 19 mμ. The antibody can not be a sphere, but rather rod-shaped, the length must be longer than 19 mμ and it must attach itself to the bacteria at its narrow edges (Fig. 2).

Neurath (10) estimated the size of the antibody in rabbit serum on the basis of the results obtained by the ultracentrifuge as the length 27.4 mμ, the breadth 3.7 mμ. From these values, the binding surface area of the antibodies is calculated in two cases, the antibody attaches itself to the bacteria at its narrow edges or at its side, as written in the seventh and eighth column of Table 3. The binding surface of the antibodies is much

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larger than the whole surface of the bacteria if they attach at their side. But in the serum dilution 1:2.5 or 1:5.0, the sum of the binding surface of the antibodies attaching to the bacteria at the narrow edges is almost equal to the whole surface of the bacteria. The size of the antibody suggested by Neurath agrees fairly well with our experimental results. Stanley's electron-microscope studies (11) of the mosaic virus and its antibody compound suggest that the length of the antibody is about 30 mμ and it attaches to the virus with its narrow edge. From Heidelberger's report (7) concerning pneumococcus and its antibody in horse serum, the same calculations are performed and shown in Table 4. Neurath estimated 95 mμ and 4.7 mμ as the length and breadth of the antibody against Pneumococcus polysaccharid in horse serum. On the basis of these values, the fifth and sixth column were calculated. These results equally suggest that the horse antibody attaches to the bacteria with its narrow edges.

3) The valency of the antibody. Bordet, Eagle, Hooker and Boyd, and Haurowitz assumpted that the antibody is monovalent, but Marrack (2) and Heidelberger (6) multivalent. Recently Pauling (12) advanced that it must be bivalent. I have already infered repeatedly that the antibody can never be monovalent. In the case of the agglutination, the bacteria are much larger than the antibodies, so the antibodies can not attach to more than two bacteria. That is, the valency of the agglutinin can not be larger than two. Moreover our results suggest that the antibody must be rod-shaped, binding with its narrow edges. As the antibody is rod-shaped, then it has only two narrow edges, where it attaches to the bacreria. The antibody must therefore be bivalent.

SUMMARY

1) The agglutinin could be estimated by measuring the nitrogen content of the specific compound of Staphylococci and their antibodies. The nitrogen content of the agglutinin is same as that of the ordinary protein.

2) From the relation of the surface area of the bacteria and the quantity of the binding agglutinin the following conclusion was induced.

I. The quantitative relation aggrees fairly with Marrack's Lattice theory but opposits to the old theory of antibody coating.

II. The antibody in the rabbit serum must be rod-shaped and bind the bacteria with its narrow edges. Its length must be larger than 19 mμ.

III. The antibody must be bivalent.

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Table 1. The nitrogen content of the precipitates.

Kind of serum	Serum dilution	Volume of the diluted serum	Volume of the bacterial suspension	Nitrogen in the precipitates	Mean
Anti-staphylococcus serum	1:10	4	1	0.614 mg	0.612 mg ± 0.006 mg
	10	4	1	0.616	
	10	4	1	0.608	
	10	4	1	0.610	
Anti-cholera serum	10	4	1	0.458	0.469 mg ± 0.012 mg
	2.5	4	1	0.476	
Anti-typhoid serum	10	4	1	0.453	
	2.5	4	1	0.485	
Normal rabbit serum (No. 1)	10	4	1	0.486	
	2.5	4	1	0.461	
Normal rabbit serum (No. 2)	10	4	1	0.467	
Physiological saline	—	4	1	0.475	0.473 mg ± 0.004 mg
	—	4	1	0.473	
	—	4	1	0.474	
	—	4	1	0.469	

Table 2. The nitrogen Content of the agglutinin.

The weight of the precipitate A	The weight of the bacteria B	The weight of the antibody in the precipitate $A-B$	The nitrogen of the antibody in the precipitate	$\frac{C}{A-B}\times 100$
45.9 mg	37.1 mg	8.8 mg	1.15 mg	13 %
34.2 mg	29.7 mg	3.5 mg	0.50 mg	14 %

Table 3-1. The antibody nitrogen in the precipitates. And the quantitative relation between the surface area of the bacteria and the binding agglutinin.

No. 1	Serum dilution	Agglutination	Agglutinin in the supernatant	Antibody nitrogen in the precipitate	The whole anti-body-N in the reacting mixture	Number of the antiboby molecules in the precipitate	Sum of the surface area of the narrow edges of the antibody	Sum of the surface area of the side of the antibody
Exp. 1. Antistaphylococcus serum No. 1. The bacterial number $=21\times10^9$. The nitrogen of the bacteria $=0.472$ mg. The whole bacterial surface $=360$ cm^2								
1	1:2.5	###	+	0.136 mg	1.68 mg	33.2×10^{14}	365 cm^2	3320 cm^2
2	5	###	+	0.136	0.84	33.2	365	3320
3	10	###	+	0.113	0.42	27.6	304	2760
4	20	###	+	0.087	0.21	21.2	233	2120
5	40	###	+	0.061	0.105	14.9	164	1490
6	80	###	+	0.051	0.053	12.4	136	1240
7	160	###	−	−	0.026	−	−	−
8	320	###	−	0.013	0.013	3.2	35	320
9	640	###	−	−	0.0065	1.6	17	160
10	1280	##	−	−	0.0033	0.8	9	80
11	2560	+	−	−	0.0016	0.4	4	40
12	5120	−	−	−	−	−	−	−

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Exp. 2. Antistaphyloccus serum No. 2.
The bacterial number $=15.2\times10^9$ The bacterial $-N=0.342$ mg. The whole bacterial surface $=260$ cm²

1	1: 5	###	+	0.086 mg	1.16 mg	21.0×10^{14}	231 cm²	2100 cm²
3	10	###	+	0.090	0.58	22.0	242	2200
4	20	###	+	0.082	0.29	20.0	220	2000
5	40	###	+	0.079	0.145	19.3	212	1930
6	80	###	+	0.055	0.073	13.4	147	1340
7	160	###	+	0.034	0.036	8.3	91	830
8	320	###	−	0.014	0.018	3.5	39	350
9	640	###	−	−	0.009	2.2	24	220
10	1280	###	−	−	0.0045	1.1	12	110
11	2560	+	−	−	0.0023	0.55	6	55
12	5120	−	−	−	−	−	−	−

Exp. 3. Antistaphylococcus serum No. 2.
The bacterial number $=17.4\times10^9$ The bacterial nitrogen $=0.454$ mg
The whole bacterial surface $=300$ cm².

1	1:2.5	###	+	0.120 mg	2.32 mg	29.3×10^{14}	323 cm²	2930 cm²
2	5	###	+	0.104	1.16	25.4	279	2540
3	10	###	+	0.091	0.58	22.2	244	2220
4	20	###	+	0.069	0.29	16.8	188	1680
5	40	###	+	0.051	0.145	12.4	136	1240
6	80	###	+	0.052	0.073	12.7	140	1270
7	160	###	+	0.038	0.036	9.3	102	930
8	320	###	−	0.041	0.018	10.0	110	1000
9	640	###	−	−	0.009	2.2	24	220
10	1280	###	−	−	0.0045	1.1	12	110
11	2560	+	−	−	0.0023	0.55	6	55
12	5120	−	−	−	−	−	−	−

* The molecular weight of the agglutinin is assumpted as 156000.

Table 3-2. The antibody nitrogen in the precipitates.
And the quantitative relation between the surface
area of the bacteria and the binding agglutinin

No. 1	Serum dilution	Agglu-tination	Agglutinin in the super-natant	Antibody nitrogen in the precipitate	The whole anti-body-N in the racting mixture	Number of the antibody molecules in the precipitate	Sum of he surface area of the narrow edges of the antibody	Sum of the surface area of the side of the antibody

Exp. 4. Antistaphylococcus serum (No. 2).
The bacterial number $=26.5\times10^9$ The bacterial $-N=0.570$ mg.
The bacterial whole surface area $=460$ cm².

1	1:2.5	###	+	0.187mg	2.32 mg	45.6×10^{14}	501 cm²	4560 cm²
2	5	###	+	0.183	1.16	44.6	490	4460
3	10	###	+	0.138	0.58	33.7	370	3370
4	20	###	+	0.105	0.29	25.6	281	2560
5	40	###	+	0.066	0.145	16.1	177	1610
6	80	###	+	0.050	0.073	12.2	134	1220
7	160	###	−	−	0.036	−	−	−
8	320	###	−	0.007	0.018	1.7	19	170
9	640	###	−	−	0.009	2.2	24	220
10	1280	##	−	−	0.0045	1.1	12	110
11	2560	−	−	−	0.0023	0.55	6	55
12	5120	−	−	−	−	−	−	−

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Exp. 5. Antistaphylococcus serum (No. 2)
The bacterial number = 39.9×10^9. The bacterial-N = 0.856 mg.
The wholet bacterial surface = 69 cm².

1	1:2.5	###	+	0.264 mg	2.32 mg	$64.4\times1^{.14}$	708 cm²	6440cm²
2	5	###	+	0.225	1.16	54.9	604	5490
3	10	###	+	0.192	0.58	46.8	514	4680
4	20	###	+	0.133	0.29	32.5	356	3250
5	40	###	+	0.063	0.145	15.4	169	1540
6	80	###	+	—	0.073	—	—	—
7	160	###	—	—	0.036	8.8	96	880
8	320	###	—	—	0.018	4.4	48	440
9	640	##	—	—	0.009	2.2	24	220
10	1280	±	—	—	0.0045	1.1	12	110
11	2560	—	—	—	0.0023	0.55	6	55

Exp. 6. Antistaphylococcus serum (No. 3)
The bacterial number = 20×10^9. The bacterial N = 0.363 mg.
The whole bacterial surface = 340 cm².

1	2.5	###	+	0.124 mg	4.52 mg	30.3×10^{14}	333 cm²	3030 cm²
2	5	###	+	0.116	2.26	28.3	311	2830
3	10	###	+	0.107	1.13	26.1	287	2610
4	20	###	+	0 085	0.565	20.7	227	2070
5	40	###	+	0.067	0.233	16.3	179	1630
6	80	###	+	0.041	0.116	9.9	108	990
7	160	###	+	0.032	0 058	7.8	86	780
8	320	###	±	0.016	0.029	3.9	43	390
9	640	###	—	—	0.0145	3.5	39	350
10	1280	###	—	—	0.0073	1.8	20	180
11	2560	##	—	—	0.0036	0.9	10	90
12	5120	±	—	—	0.0018	0.45	5	45
13	10240	—	—	—	—	—	—	—

Table 4. Quantitative relation of the bacterial surface area*
and the binding agglutinin calculated from
Heidelberger's report.

The bacterial nitrogen	The whole (1) bacterial surface	The agglutinin nitrogen	The number of agglutinin molecules	The sum of the surface area of the narrow edges of antibodies	The sum of the side surface of antibodies
0.112 mg	83–530 cm²	0.17 mg	7.0×10^{14}	120 cm²	3200 cm²
0.244	180–1100	0.24	9.8×10^{14}	170	4400
0.366	280–1800	0.34	14×10^{14}	250	6300
0.488	370–2400	0.41	17×10^{14}	300	7700
0.732	550–3500	0.56	23×10^{14}	400	10300
0.976	635–4700	0.59	24×10^{14}	420	11000
1.220	910–5900	0.61	25×10^{14}	440	11000
1.464	1095–7000	0.60	25×10^{14}	440	11000

* J. Exp. Med., 65 890 (1937). Table 5.

(1) In the report, J. Exp. Med., 63, 737 (1936), 0.44 mg the bacterial nitrogen conform to 44×10^9 the bacterial number. From this value and the bacterial diameter 0.5–1.25 μ (assumpted) the bacterial whole surface area was calculated.

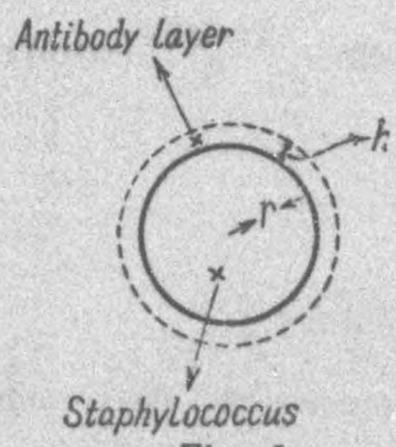

Fig. 1.

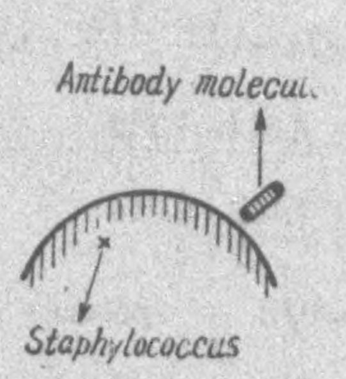

Fig. 2

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STUDIES ON THE POTENCY TEST OF TUBERCULIN

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To find the potency of tuberculin, the local reactions produced by graded intradermic injections of the standard and the test tuberculin are compared, but this international method can only suggest whether the test tuberculin differs appreciably from the standard or not. Now we could find the calculation method to know how much the potency of the test tuberculin differed from the standard.

From our many experimental results we found the following relation between the potency of tuberculin (p) and the diameter (d) of the skin redness.

$\sqrt{d} = \alpha + \beta \log p$ (α and β are constants). This relation exists not only in skin test by guinea pig, but also by human cases, for example in old Pirque's data.

On the basis of this relation the potency of test tuberculin can be calculated as following.

Table 1

Guinea pig No.		Dilution			
		1:500	1:1000	1:2000	1:4000
1	Diameter of the redness of the standard (mm)	10×10	6×7	4×4	—
	Diameter of the redness of the test tuberculin (mm)	7×7	5×5	3×3	—
:	:	:	:	:	:

For example, if the results in table 1 is obtained, instead of the dilution such 1:500 etc, we use their logarithm as 2, 1, 0 and -1, and we make the next table and then calculate.

Standard

X	Y^2	Y	XY
2	10.00	3.162	6.324
1	6.50	2.550	2.550
0	4.00	2.00	0
3	20.500	7.712	8.874

$$S_x = 2^2+1^2+0^2-\frac{(2+1)^2}{3} = 2$$

$$Cxy = 8.874 - \frac{3\times 7.712}{3} = 1.162$$

Test

X	Y^2	Y	XY
2	7.00	2.646	5.292
1	5.00	2.236	2.236
0	3.00	1.732	0
3	15.00	6.614	7.528

$$S_x = 2$$

$$Cxy = 7.528 - \frac{3\times 6.614}{3} = 0.914$$

$$\log_2\theta = \frac{\frac{6.614-7.712}{3}}{\left(\frac{1.162+0.914}{2+2}\right)} = -0.7086$$

$\theta = 0{,}61 \ldots\ldots$ potency of the test tuberculin

Usually many data are obtained from several animals and from such data the most probable value can be calculated according to the following relation.

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$$\log\theta = \frac{(\bar{X}_s - \bar{X}_t) + (\bar{Y}_t - \bar{Y}_s)}{\left(\frac{\Sigma Cxyt + \Sigma Cxys}{\Sigma Sxt - \Sigma Sxs}\right)} = \frac{\Sigma Cxyt + \Sigma Cxys}{\Sigma Sxt + \Sigma Sxs}$$

$\bar{X}_s$.... Mean of the logarithm of the dilution of the standard.
$\bar{X}_t$.... Mean of the logarithm of the dilution of the test.
$\bar{Y}_t$.... Mean of the root of diameter of the redness of the test.
$\bar{Y}_s$.... Mean of the root of the diameter of the standard.
$\Sigma Cxyt$.... Sum of the Cxy of the test.
$\Sigma Cxys$.... Sum of the Cxy of the standard.
ΣSxt .... Sum of the Sx of the test.
ΣSxs .... Sum of the Sx of the standard.
Usually, $\bar{X}s - \bar{X}t = 0$ and $\Sigma Sxt = \Sigma Sxs$.

In one experiment in which the standard and its 1.5 times diluted solution were compared, as // the value 0.58 was obtained. This value (0.58) is not so different from the theoretical value ($0.66 = \frac{1}{1.5}$).

The regression coefficient β depends upon many experimental conditions, particularly on the season in which the test is performed. From our results β in summer was larger than in winter. And also in one experiment β of the test animals placed in warm room (27C°) was larger than that of the animals in cold room (13°C). Their difference was significant.

This report was published in detail at the Meeting in our Institute, August 1944 and here we express our sincere appreciation to Dr. M. Masuyama for his kind mathematical co-operation.

A NEW METHOD OF TESTING THE POTENCY OF TUBERCULIN

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As described in the previous report (1), there is the relation between the root of the redness, d, and the potency of the tuberculin, p,

$\sqrt{d} = \alpha + \beta \log p$ (α and β are constants),

and on the basis of this relation the potency of the test tuberculin can be calculated. But this method of calculation is considerably perplexing as stated in the previous report.

Now the potency of penicillin is usually measured by the cup method and the error due to this process is remarkably small, for example its standard deviation is about 2 % in our laboratory (2).

This cup method rests on the basis of the following relation (3),

$d = a + b \log c$ (d is the diameter of the inhibition zone, c the potency of penicilin, a and b are constants).

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Now there is a similarity between the potency test of tuberculin and penicillin.

In the international method of testing the potency of tuberculin several dilutions (1:500, 1:1000, 1:2000, and 1:4000) of both the test and the standard tuberculin are injected into the skin of the sensitized guinea pigs. But, if only two dilutions, for example 1:500 and 1:1000 or 1:500 and 1:2000, are injected instead of several dilutions, then from the roots of the diameters of the redness observed after twenty four hours the potency of the test tuberculin can be calculated in the same way as in the case of penicilin.

In this report this new method and the error due to this method are described.

I. The procedure of our new method.

The following dilutions of the test tuberculin (*T*) and the standard tuberculin (*S*) are made in sterile physiological saline, using sterile calibrated pipettes and calibrated flasks:

1 in 500 and 1 in 2000.

Eight or twelve sensitized guinea-pigs are epilated along both flanks by careful shaving. Each is inoculated intradermally with 0.1 cc of each dilution of the two tuberculins, the dilutions of the test tuberculin along one flank, those of the standard tuberculin in corresponding skin sites of the opposit flank as shown in Table 1.

Table 1 The inoculation of the test and the standard tuberculin.

S 500.....1 in 500 dilution of the standard tuberculin
S 2000.....1 in 2000 „ „ „
T 500.....1 in 500 dilution of the test tuberculin
T 2000.....1 in 2000 „ „ „

No. of guinea pig	Left or right side	Inoculated place			
		Near head ←		→ Near tail	
1	left	S 500	S 2000	S 500	S 2000
	right	T 500	T 2000	T 500	T 2000
2	left	S 2000	S 500	S 2000	S 500
	right	T 2000	T 500	T 2000	T 500
3	left	T 500	T 2000	T 500	T 2000
	right	S 500	S 2000	S 500	S 2000
4	left	T 2000	T 500	T 2000	T 500
	right	S 2000	S 500	S 2000	S 500
5	left	S 500	S 2000	S 500	S 2000
	right	T 500	T 2000	T 500	T 2000
6	left	S 2000	S 500	S 2000	S 500
	right	T 2000	T 500	T 2000	T 500
7	left	T 500	T 2000	T 500	T 2000
	right	S 500	S 2000	S 500	S 2000
8	left	T 2000	T 500	T 2000	T 500
	right	S 2000	S 500	S 2000	S 500

If twelve animals are used, then the other four animals are inoculated in the same way as No. 1–4 animals in Table 1.

The diameters of the redness are measured approximately 24 hours after inoculation.

From the diameters of the redness observed, the potency is calculated in the following way:

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The diameters of the redness induced by S 500 .....$a1$, $a2$, ... an.
The diameters of the redness induced by S 2000.....$b1$, $b2$, ... bn.
The diameters of the redness induced by T 500 .....$c1$, $c2$, ... cn.
The diameters of the redness induced by T 2000.....$d1$, $d2$, ... dn.
The potency of the test tuberculin .....θ

then

$$\log\theta=\frac{(\sum\sqrt{c}+\sum\sqrt{d})-(\sum\sqrt{a}+\sum\sqrt{b})}{(\sum\sqrt{c}+\sum\sqrt{a})-(\sum\sqrt{d}+\sum\sqrt{b})}\times\log 4$$

$$(\sum\sqrt{a}=\sqrt{a_1}+\sqrt{a_2}+\cdots\cdots+\sqrt{a_n})$$

The calculation can be eliminated, by using the nomogram (3) for the potency test of penicillin. And the root of the diameters can be easily obtained by Barlow's (4) table.

If instead of two dilutions (1 in 500 and 1 in 2000) the other two dilutions such as 1 in 500 and 1 in 1000 are injected, then the potency of the test tuberculin can be calculated as follows:

$$\log\theta=\frac{(\sum\sqrt{c}+\sum\sqrt{d})-(\sum\sqrt{a}+\sum\sqrt{b})}{(\sum\sqrt{c}+\sum\sqrt{a})-(\sum\sqrt{d}+\sum\sqrt{b})}\times\log 2$$

but in this case

$b1$, $b2$, ... bn = The diameters of the S 1000
$d1$, $d2$ ..... dn = The diameters of the T 1000.

In this case the other nomogram used in the potency test of penicillin is also available.

II. The error due to this new method.

Owing to want of animals, the experiment to find the error of this method could not be performed. But from the many results obtained by the past international method the error due to our new method can be estimated.

In the international method several dilutions of the test and the standard tuberculin are inoculated as shown in Table 2.

Table 2. The inoculation of tuberculin by the international method.

No. of guinea pig	Left of right side	The inoculated place			
		near head		near tail	
1	left right	S 500 T 500	S 1000 T 1000	S 2000 T 2000	S 4000 T 4000

S 500.....1 in 500 dilution of the standard tuberculin.
S 1000.....1 in 1000 „ „ „
S 2000.....1 in 2000 „ „ „
S 4000.....1 in 4000 „ „ „
T 500.....1 in 500 dilution of the test tuberculin.
T 1000.....1 in 1000 „ „ „
T 2000.....1 in 2000 „ „ „
T 4000.....1 in 4000 „ „ „

If we considered S 500, T 500, and S 2000, T 2000 as the standard and S 1000, T 1000, and S 4000, T 4000 as the test tuberculin, then the theoretical potency of the test tuberculin is 0.50.

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On the other hand it can be calculated from the redness observed as in the following way:

Diameter of the redness of S 500 ..... $a1$
Diameter of the redness of T 500 ..... $a2$
Diameter of the redness of S 2000 ..... $b1$
Diameter of the redness of T 2000 ..... $b2$
Diameter of the redness of S 1000 ..... $c1$
Diameter of the redness of T 1000 ..... $c2$
Diameter of the redness of S 4000 ..... $d1$
Diameter of the redness of T 4000 ..... $d2$

$$\text{then } \log\theta=\frac{(\sqrt{c_1}+\sqrt{c_2}+\sqrt{d_1}+\sqrt{d_2})-(\sqrt{a_1}+\sqrt{a_2}+\sqrt{b_1}+\sqrt{b_2})}{(\sqrt{c_1}+\sqrt{c_2}+\sqrt{a_1}+\sqrt{a_2})-)\sqrt{d_1}+\sqrt{d_2}+\sqrt{b_1}+\sqrt{b_2})}\times\log 4$$

In this way the potency of the test tuberculin observed in each animal was calculated and is described in Table 3. The mean of the potencies is 0.515 and it agrees fairly well with the theoretical value 0.50.

Table 3. The potency of the test tuberculin (the theoretical value is 0.50) calculated in each animal.

Exp. No.	Animal No.	Potency
1	1	0.421
	2	0.530
	3	0.593
	4	0.467
2	5	0.558
	6	0.253
	7	0.557
	8	0.602
3	9	0.644
	10	0.560
	11	0.524
	12	0.453
4	13	0.340
	14	0.625
	15	0.595
	16	0.402
5	17	0.443
	18	0.494
	19	0.544
6	20	0.544
	21	0.415
	22	0.347
7	23	0.524
	24	0.468
	25	0.597
8	26	0.450
	27	0.480
9	28	0.387
	29	0.715
	30	0.597
10	31	0.605
	32	0.744
	33	0.445
	34	0.470
	35	0.564
11	36	0.432
	37	0.525
	38	0.678
	39	0.642
	40	0.544
12	41	0.539
	42	0.544
	43	0.606
	44	0.556
	45	0.543
13	46	0.478
	47	0.601
	48	0,343
	49	0.393
14	50	0.431
	51	0.554
	52	0.438
Sum	52	26.801

$$\text{potency}.....\frac{26.801}{52}=0.515$$

From the results shown in Table 3, the variance $u(5)$ is obtained as follows:

$$u^2=\frac{1}{52-1}\left\{(0.421^2+0,530^2+\cdots\cdots+0.438^2)-\frac{(26.801)^2}{52}\right\}=0.009965$$